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APPLICATION NUMBER: *60/538,450*
FILING DATE: *January 22, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/02087*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Rev 01/04

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (c).

MAIL STOP PROVISIONAL APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Docket Number: INCOM-001Xq800	Type a Plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

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Detarando	Michael	A.	Sturbridge, Massachusetts
Kass	Jason		Auburn, Massachusetts

[ ] Additional Inventors are being named on Page 2 attached.

TITLE OF THE INVENTION (280 characters max)

MICROARRAY PLATE WITH LARGE DIAMETER, HIGH RESOLUTION WELLS, MICROFLUIDIC FIBER OPTIC INTERROGATED MICROWELL BIOCHIPS

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(CHECK ALL THAT APPLY)*

[X] Specification Number of pages [72]

[ ] Drawing(s) Number of sheets [ ]

[X] Small Entity status is entitled to be, and hereby is, asserted for this application

[ ] Other (specify)

METHOD OF PAYMENT *(CHECK ONE)*

[X] A check in the amount of $80.00 is enclosed to cover the Provisional Filing Fee

[ ] The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 23-0804

Please recognize the following attorneys with powers in this application.

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PROVISIONAL APPLICATION FILING ONLY

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U.S. PROVISIONAL APPLICATION

ENTITLED

MICROARRAY PLATE WITH LARGE DIAMETER, HIGH RESOLUTION WELLS, MICROFLUIDIC FIBER OPTIC INTERROGATED MICROWELL BIOCHIPS

BY

MICHAEL J. MINOT

MICHAEL A. DETARANDO

JASON KASS

EXPFRESS MAIL NO: EV 009947057 US

1	Proposal Submitted By (Please use legal name) Full First Name, Middle, Last Dr. Michael J. Minot			Employee No. NA
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2	Proposal Submitted By (Please use legal name) Full First Name, Middle, Last Michael A. Detarando			Employee No.
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*3	Proposal Submitted By (Please use legal name) Full First Name, Middle, Last Jason Kass			Employee No.
	Organization (Unit/Div./Dept./Section) Incom Inc.	Electronic Mail Address jlk@incomusa.com	Bldg. No./Mail Stop	Extension
* If space for additional submitters is required, please use another sheet and attach any supplementary comments.				
Manager		Electronic Mail Address	Bldg. No./Mail Stop	Name of Program and Technology

Descriptive Title of Proposal:

MicroArray Plate with Large Diameter, High Resolution Wells, Microfluidic Fiber Optic Interrogated Microwell Biochips

Provide a brief summary or abstract of the invention, specifically pointing out the features you think are new.

An epoxy based photo resist coating is applied to an optical fiber array substrate and then exposed through a photo mask to form the desired pattern of wells. After exposure, the coating is selectively removed to reveal the wells. The remaining coating material is then cured, to form a hard layer which becomes an intimate part of the final product. Well walls consist of the cured epoxy coating material. The fiber optic array substrate material forms the well bottoms.

One advantage of the process is the ability to form wells on the surface of a fiber optic array without having to etch the glass. Another advantage is to allow the formation of flow control channels that insures that sequencing reagents flow uniformly across the micro well plate. The use of a deposited permanent photo resist coating also make it possible to create large diameter wells (100μ diameter or larger) onto a fiber optic array with an arbitrary fiber diameter. This enables, for example, the fabrication of a MicroArray Plate with large diameter; high resolution wells. For example, a technique for producing 100μ diameter wells with many 3μ diameter fibers on the bottom was demonstrated.

Provide here, using added pages, a more detailed technical description of your invention, including the advantage(s) and the problem(s) solved by the invention, and how each is accomplished. Please indicate the current methods or techniques used to solve the problem(s) and the deficiencies of these methods or techniques. Sketches, drawings, notebook pages, memos or photos can be very helpful and should be attached if possible.

SEE SBIR PROPOSAL WRITE-UP FOR ADDITIONAL DETAILS

Fiber optic microarray plates are advantageous for various kinds of biological and chemical analysis, since

the reaction occurring inside the well can be monitored (interrogated) through the optical fiber that is an integral part of the well. The optimal dimensions for wells in a Fiber Optic Microarray plate depend on what is being analyzed. For example, the optimal well size for bacteria, might be considerably smaller compared to the optimal well size for mammalian cells, or genomic or proteomic material.

In many applications, well diameter corresponds to the diameter of the fiber in the array. The fiber is processed to remove some core material, forming the well with a bottom consisting of the remaining portion of the fiber. For example, a fiber optic array plate is fabricated with fibers having a core diameter of 42 microns, surrounded by cladding glass so that the fibers are 50μ center to center. Clad glass is selected to be more chemically resistant compared to the core glass, allowing selective removal of the core glass, while leaving the clad glass intact. The core glass can be chemically removed to form wells of various depths. For example, the well depth might be 55μ. Reactions occurring in each well can be monitored through the individual fibers forming the bottoms for each well. This example might be referred to as the 'one well, one fiber' scenario.

In other instances, it is desirable to have a large diameter well, with many small diameter fibers forming the bottom. This would allow the reaction process occurring in the large diameter well to be monitored more discretely, with higher geometric resolution across the well. For example, it might be desirable to have 100μ diameter (or larger) well diameter, with multiple 3μ fibers forming the well bottom (one well, multiple fiber scenario).

One approach to produce this type structure would be to select the core and clad glass compositions so that **both** could be etched away to form the 100μ wells. Photo resist or other masking strategies would have to be used to prevent areas between wells from etching. Accomplishing this in practical application would be difficult since most mask materials do not tolerate the time and temperature conditions used to etch the glass. Furthermore, it would be difficult to prevent an undercutting of the mask material if the clad glass were readily chemically soluble.

A different approach would be to overlay or laminate a thin layer of material (glass or plastic material) with 100μ holes already formed onto an optical fiber array having 3μ cores. No etching would be involved in this scenario. This technique would be feasible however difficulties are anticipated handling the (very) thin layer to be laminated. Furthermore, achieving good adhesion in this scenario might be difficult.

The preferred option would be to use a non-removable photo resist coating which could applied to an optical fiber array and be then patterned to form 100μ holes. The photo resist coating would become an intimate part of the final coating. The detail procedure would be as follows:

The proposed scenario is based on using SU-8 or SU-8 2000 photo resist manufactured by MicroChem Corporation of Newton MA. SU-8 (formulated in GBL) and SU-8 2000 (formulated in cyclopentanone) are chemically amplified; epoxy based negative resists. They are generally believed to have good adhesion properties to glass. Standard formulations are offered to cover a wide range of film thicknesses from <1μm to >200μms. SU-8 and SU-8 2000 resists have high functionality, high optical transparency and are sensitive to near UV radiation. Images having exceptionally high aspect ratios and straight sidewalls are

readily formed in thick films by contact-proximity or projection printing. Cured SU-8 is highly resistant to solvents, acids and bases and has excellent thermal stability, making it well suited for applications in which cured structures are a permanent part of the device.

A glass fiber optic array substrate is fabricated in the shape of a circular disc of appropriate thickness, to facilitate the application of the liquid resist using standard spin application. All processing is done under yellow lights selected to prevent premature exposure of the photo resist. Detail processing steps included in the attached product specifications. The overview is as follows:

1. Substrate Pretreatment,
2. Coat,
3. Soft Bake,
4. Expose
5. Post Expose Bake
6. Develop
7. Rinse & Dry
8. Hard Bake / Cure

After exposure, the coating is selectively removed, or developed, to reveal the desired pattern of wells. The remaining coating material is then cured, to form a hard layer which becomes an intimate part of the final product. Once the hard bake / cure has been completed, the cross-linked epoxy coating is extremely difficult to remove.

This new technique for producing fiber optic interrogated microwell plates offers a number of advantages:

- One advantage of the process is the ability to form wells on the surface of a fiber optic array without having to etch the glass. This results in significant cost reduction, since the glass etching process including disposal of hazardous materials, adds considerably to the cost of the finished product.

- The old process can only be advantageously applied to certain glass compositions that have the optical properties that are desirable (high numerical aperture =1) and which can be etched to selectively remove core glass without removing clad glass. Furthermore, not all glass compositions are able to be etched without leaving residual surface films or well morphology that is not optimal for this application. The new process overcomes this disadvantage by decoupling issues associated with glass composition with the well forming step. This opens up the potential of creating micro fluid wells on the surface of fiber optic array plates made from glass compositions that are not suitable for etching and that are inherently lower cost compared to others, resulting in a considerable cost reduction.

- The new process makes it possible to decouple well dimensions from the diameter of the fiber used in the fiber optic array. Well diameter and depth can be optimized to the biological sample being tested, while the fiber diameter of the underlying substrate can be selected to optimize the dimensional requirements of optical detection system being used. For example, it might be desirable to create large diameter wells (100μ diameter or larger) onto a fiber optic array with 3μ diameter fibers.

Initial Feasibility Trials: Tuesday, December 9, 2003, done at MicroChem Corporation, (1254 Chestnut Street Newton, MA 02464). Participating was Michael Minot, MinoTech Engineering, Jason L. Kass, Incom Inc., and Robert Hardman and Jeff Walling from MicroChem. A series of feasibility trials were done using a photomask test pattern provided by MicroChem. Incom provided two types of glass substrate for the trials:

- Window Glass – cut into 2 inch diameter discs ground and polished to a nominal 1 mm thickness.
- 3 micron fiber optic faceplates – standard Incom faceplate material (MDH – X14, C-5 core and clad glass compositions) fabricated into 2 inch discs ground and polished to a nominal 1 mm thickness.

Trials using window glass were included, since we anticipated the need to learn proper techniques to spin deposit the photo resist material to the appropriate thickness, as well as to find exposure levels that were appropriate for our application.

The photo mask provided by MicroChem had a variety of test patterns including some that closely approximated structures of interest. Specifically, there were hexagonal cells with different dimensions:

1) 150 micron on 200 micron centers.
2) 120 micron on 160 micron centers.
3) 300 micron on 400 micron centers.

Feasibility trials evaluated these variables:

1) Different photo resist materials: SU-8, SU-8 25, SU-8 2025
2) Adhesion promoters intended to enhance the bond between substrate and photo resist: None, HMDS, XP Omni.
3) Substrate cleaning procedures.
4) Photo resist thickness (55 micron target)
5) UV exposure level: 100 mJ to 250 mJ

A total of six samples were prepared, four on window glass and two on 3 micron fiber optic faceplate glass. The trials on window glass were useful in establishing spin rates to achieve the target film thickness, however film adhesion was not adequate despite trying different adhesion promoters. Samples #5 and #6 used the 3 micron fiber array faceplate as substrate. Photo resist SU-8-2025 was applied at 1100 RPM. UV exposure for sample six was increased to 250 mJ, based on the poor adhesion of earlier samples, the overall film thickness, and the fact that glass substrates often require more exposure since little UV is reflected back

through the coating compared to silicon surfaces.

The photo resist test pattern on sample #6 was well adhered. The film thickness was measured at 56-57 microns thick.

Three photos were taken under the microscope. The are attached to this document and a re labeled as follows;

Photo #1 – 120 microns cell, 150 microns on center @ 10 X 10 magnification.

Photo #2 – 120 microns cell, 150 microns on center @ 20 X 10 magnification.

Photo #3 – 120 microns cell, 150 microns on center @ 10 X 10 magnification.

Witnessed and Understood By	Date
Submitter(s) Signature(s) Michael J. Minot	Date Initially prepared 10/28/03 and subsequently edited.

Names of others known to have worked on this or a similar invention.

Identify any known similar or related Invention Proposals, patents or publications, Incom Inc. or MinoTech Engineering commercial products, or indicate none.

NONE (Fiber optic interrogated microwells for biosensor applications are known. Use of SU-8 to optically pattern permanent microstructure on glass or silicon is also known, and has been used for other biochip applications. We believe this is the first instance of combining fiber optic arrays as a substrate with SU-8 formed microstructure, to form fiber optic interrogated microfluidic devices.)

Has a model, a prototype or experiment of the invention been built, made, run or tested? Yes __X__ No ___
Initial feasibility trials have been completed.

Is the invention used in a current product(s) or planned for use in a future product(s)? Yes _X_ No ___
If so, please identify the program(s) or product(s) and introduction dates.

An SBIR funding proposal is being prepared and is due by January 20, 2004. In anticipation of this deadline we would like to be able to discuss development plans with Micro-Chem Corporation, in order to engage their support for some of the work. In addition, we would like to discuss the technology with 454 Corporation, and BioTrove Corporation, since both are prospective customers for the technology.

Plans are also underway to prepare samples for evaluation as part of informal development collaboration with Dr. David Walt, of Tufts University.

Indicate the date(s) of any previous or planned future disclosure external to Incom Inc. or MinoTech Engineering (has the invention been disclosed or is it planned for disclosure outside of either organization) and identify the type of disclosure (by agreement, demonstration, paper or presentation given, market probe, published article, etc., and if convenient, please provide a copy of the agreement, paper or article).

Michael J. Minot, Michael A. Detarando, and Jason L. Kass met with Dr. David R. Walt, at Tufts University on Tuesday October 7, 2003. Dr. Walt provided guidance regarding the kind of microarray samples that might be useful experimentally to him. These included:

1. **Bacteria microscope slides** –3μ-diameter wells processed to achieve 3μ well depth.
2. **Mammalian Cell microscope slides** – with 25μ-fiber pitch, and with 21μ-core diameter and 10μ well depth.
3. **100μ (or larger) Diameter Wells with 3μ Resolution** – Different approaches were discussed to achieve this design concept including coating or laminating a thin layer of material with 100μ holes onto an optical fiber array having 3μ cores.

Source of outside funding, if any.

This development program is being paid for by Incom Inc.

Witnessed and Understood By	Date

Submitter(s) Signature(s)	Date
Michael J. Minot	Initially prepared 10/28/03 and subsequently edited.

1. Problem addressed or function provided by the invention.
Example 1A: Finisher cost reduction.

This new technique for producing fiber optic microarrays offers a number of advantages:

- Integrated Microfluidic Structures - The same method of depositing a permanent epoxy based photo resist to form structures such as analysis wells on a fiber optic array plate by layer can also be used to implement other more complex microfluidic structures, such as loading ports, mixing and reaction reservoirs, and flow channels. This can be used to enhance the ability to flow sequencing reagents uniformly across the entire surface of the plate which can have a significant impact on the sensitivity on the resolution and sensitivity of the diagnostic technique. In addition to improving flow uniformity, micro channels formed on the surface of the fiber optic faceplate can be used to separate the flow of reagents so that multiple sequencing reagents can be flowed across different parts of the biochip at the same time. For example, if the normal test calls for sequentially flowing 4 reagents, each separated by a rinsing step, the time required to complete the analysis can be significantly reduced by partitioning the biochip into distinct regions with its own micro channel flow, and flowing all 4 reagents across these different sections of the array, simultaneously.

- Cost Reduction: One advantage of the process is the ability to form wells on the surface of a fiber optic array without having to etch the glass. This results in a significant cost reduction, since the glass etching process including disposal of hazardous materials, adds considerably to the cost of the finished product.

- Applicable to All Glass Compositions: The old process could only be advantageously applied to certain glass compositions that had the optical properties that were desirable (high numerical aperture =1) and which could be etched to selectively remove core glass without removing clad glass. Furthermore, not all glass compositions are able to be etched without leaving residual surface films or well morphology that is not optimal for this application. The new process overcomes this disadvantage by decoupling issues associated with glass composition with the well forming step. This opens up the potential of creating micro fluid wells on the surface of fiber optic array plates made from glass compositions that are inherently lower cost compared to other, resulting in a considerable cost reduction.

- Improved Resolution by Decoupling Fiber Size from Well Dimensions: The new process makes it possible to decouple well dimensions from the diameter of the fiber used in the fiber optic array. Well diameter and depth can be optimized to the biological sample being tested, while the fiber diameter of the underlying substrate can be selected to optimize the dimensional requirements of optical detection

system being used. For example, it might be desirable to create large diameter wells (100μ diameter or larger) onto a fiber optic array with 3μ diameter fibers.

- Labeling: It is highly desirable to be ably to apply unique labels to the fiber optic micro array plate facilitating traceability of samples. Ideally, the label would be microscopic, easily read by the diagnostic instrument, permanent, and able to be applied to the micro array plate without damaging or interfering with the surface of the micro array plate. Laser scribed bar codes micro crack and damage the surface of the glass plate and are not satisfactory. Traditional inks are not well adhered to glass. Ceramic frits applied to the glass surface create a raised surface profile that interferes with sealing the plate to prevent leaks. Forming structures such as wells or channels on a fiber optic array plate by depositing a permanent epoxy based photo resist layer also makes it possible apply permanent microscopic, high resolution labels. The uncured photo resist can be irradiated with a UV laser creating an alpha numeric or symbolic label which would be a permanent part of the finished product.

Example 1B: Annotation of copies.

2. Central thrust of the invention:

Example 2A: Design incorporates fewer parts.

Example 2B: Uses low cost LCD to write annotation messages.

3. Could invention have impact beyond current description?

Example 3A: Could also function for printer finisher.

Example 3B: Could also function to erase/edit copy.

4. Potential for MINOTECH application. Specify product or technology program if possible.

Example 4A: Mainlline approach in Program Q.

Example 4B: Adds significant feature to future products.

5. Value to competitors; potential for license or trade.

Example 5A: Enables much lower cost finishing than any known system and opens possibilities of moving finishing down-market.

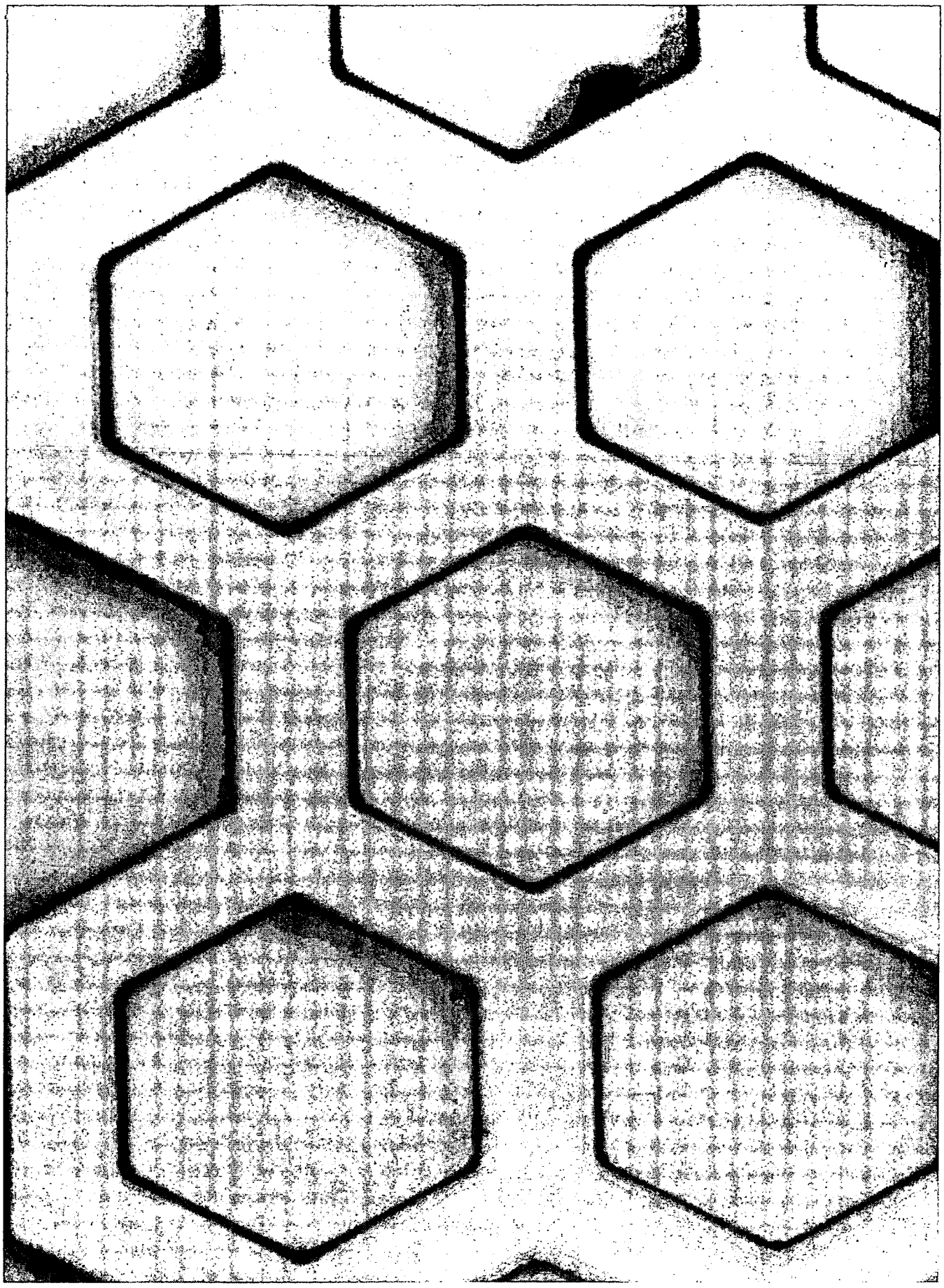
Example 5B: Low cost will be hard to match.

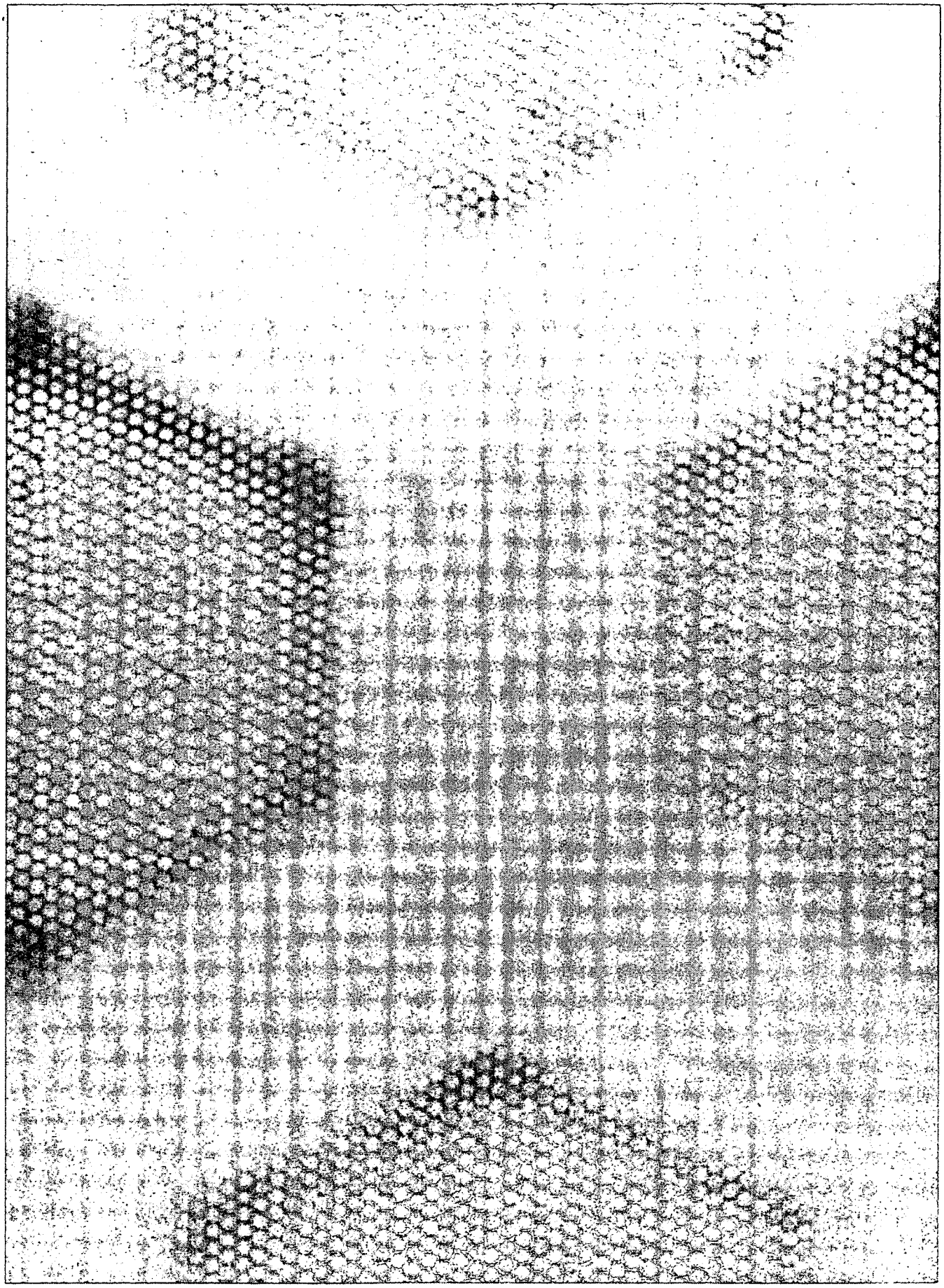
6. Please indicate any related patents, publications or activities you know of.

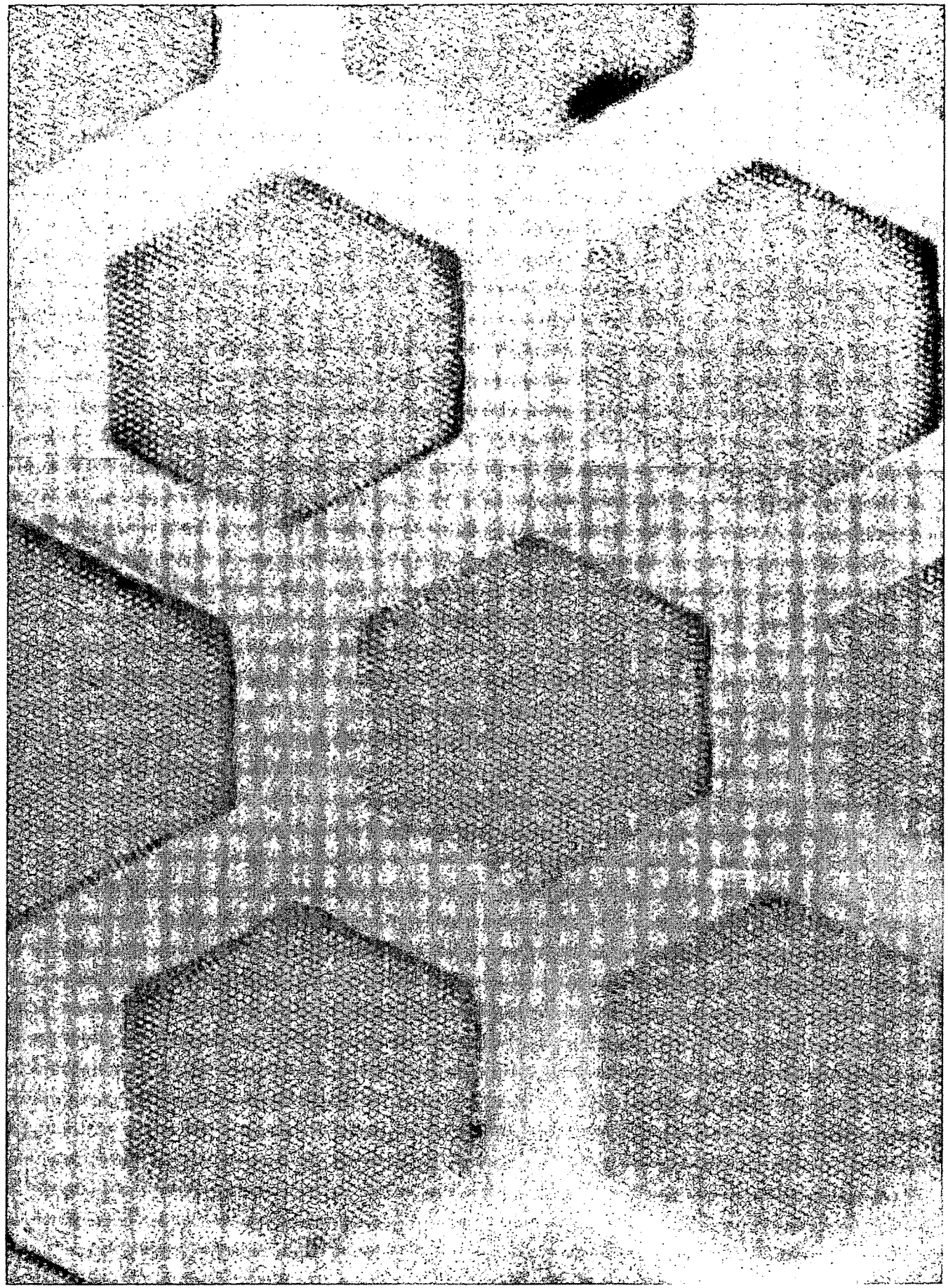
7. I would recommend the following form(s) of protection:

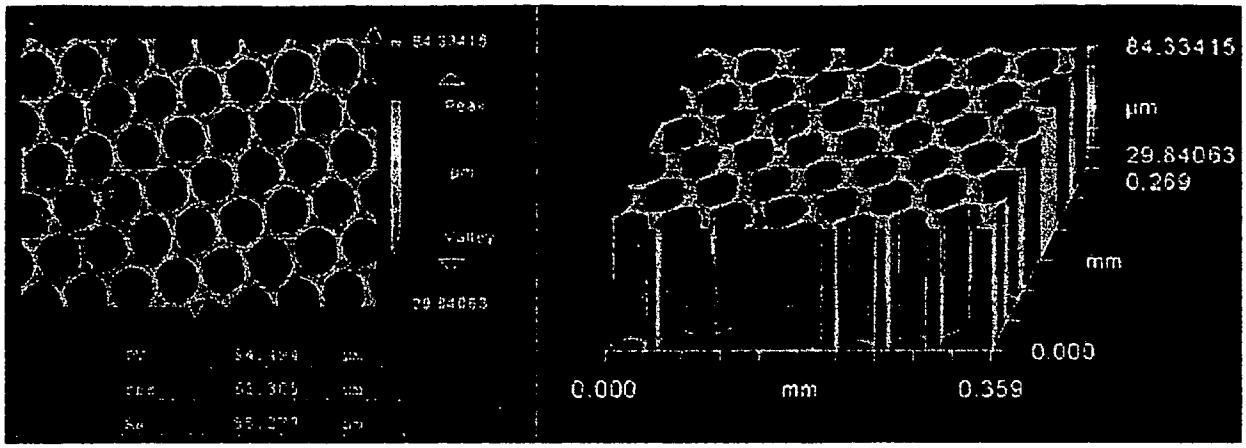
Patent ___ Defense Publication ___ Keep Trade Secret ___ None ___

Comments:








84.33415
µm
29.84063
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Microfluidic Fiber Optic Interrogated Microwell Biochips

(nomenclature: fiber optic interrogated microwell plate, fiber optic interrogated titer plate, FOI Titer Plate[tm], FOI Micro Titer Plate[tm], fiber optic interrogated microwell sensor)

I. Cover Sheet and Certification

II. Project Summary (no proprietary information)

This Small Business Innovation Research Phase I project.....targets development of fiber optic interrogated microwell biochips that combine low cost manufacturing strategies with microfluidic design features not achievable with existing manufacturing techniques.

A. Intellectual merits (200 words)

1. Identification of the problem or opportunity

DNA biochip sensors fabricated by etching high-density fiber optic arrays have smaller feature sizes and higher packing densities compared to other DNA array technologies[1]. When used in conjunction with encoded microspheres, and by coupling the fiber optic array to a CCD detection system, the shortest assay time compared to other high-density analysis techniques have been demonstrated[2]. Fiber optic array technology can be used to detect small changes in DNA sequence and has been demonstrated as a useful tool for study of single-nucleotide polymorphisms (SNPs)[3]. Etched microwells on a fiber optic array plate can serve as a platform for polymerase chain reaction amplification (PCR). A single 25 mm X 75 mm fiber optic array plate was shown to function as 370,000 discrete reaction vessels achieving high yield amplification with a total reaction volume of only 15.3μL.[4]

The full potential of these techniques will only be realized when the high density analysis offered by the fiber optic microarray can be fully integrated onto a self contained, low cost, disposable, microfluidic biochip. This desirable objective has been frustrated by the limitations of the 'etched well' manufacturing strategy used to produce these biochips. Combining photo curable epoxy based compositions that form permanent resist layers, such as SU-8, with fiber optic array technology expands the design options for microfluidic devices, while significantly reducing the cost of the biochip produced.

2. Research objectives

The objectives of this research is to develop techniques to apply permanent photocurable resist materials such as SU-8 to a fiber optic array, forming fiber optic interrogated analysis wells while eliminating the need for etching. Furthermore, the techniques developed will demonstrate capability to produce fully integrated microfluidic devices that incorporate components such as loading ports, flow control channels, mixing and reaction zones, fiber optic interrogated analysis wells, nutrient channels, and recovery ports in a low cost biochip

suitable for one time (or multiple) use. Ultimately, the objective of this research is to develop a low cost, disposable, high resolution biochip.

3. Description of the research, and anticipated results

4. Potential applications

Direct application of this technology is for biosensor micro-arrays used for DNA / genomic and proteomic materials, including living cells. The underlying technology could also be applied to a variety of sensor applications such as chemical sensing and explosive detection. This technology has broad implications for glass polymer composite micro fluidic device development and broad applications to homeland security.

B. Broader Impacts of the proposed activity

1. How will the innovation enhance scientific and technological understanding

2. Potential societal and commercial impact of the project.

Fiber optic interrogated microwell arrays provide a platform for a variety of high density, reproducible and accurate biochip sensors. A number of commercial firms (454 Corporation, Illumina Corporation, and others) and universities (Tufts University) are already developing biochip sensor products based on fiber optic interrogated microwells. Despite intensive development of the in the areas of encoding techniques, microsphere development, reagents, and informatics, little has been done to advance the development and reduce the cost of the underlying fiber optic interrogated microwell array. Consequently, the technology still largely resides in R&D labs of commercial and educational organizations.

Successful commercial transfer from R&D to the clinical laboratory will require that the fiber optic assay platform be further developed for: a) increased diagnostic speed, b) higher throughput capacity, and c) reduced manufacturing costs. The proposed technology development will result in low cost, miniaturized, fully integrated microfluidic fiber optic interrogated biochips, and will significantly accelerate the use of these methods in the clinical environment.

C. Key words

1. Areas of technical expertise in science, engineering or education

2. Application area

D. Topic name & subtopic:

Biotechnology –Biochips BT/C

III. Table of Contents

IV. Project Description (15 pages)

A. Identification and significance of the Innovation

1. Statement of the specific innovation research proposed

2. Explanation of how the innovation is relevant to meeting the needs described in the subtopic narrative:

a) BioTechnology (BT) Topic

(1) Early detection of threats posed by bioterrorism

(2) Innovative new products, devices, processes, or services

(3) Identify beneficiaries of the proposed technology

(4) Potential for commercialization

(5) R&D that results in the near-term application of a product, process, or device,

(6) R&D that greatly enhances the ability of scientists and engineers to conduct fundamental or applied research

b) Subtopic C: Biochips - "Biochips" are biologically based micro array and micro fluidic devices used for analysis and synthesis

(1) How can they be made at lower cost

(2) How can their applications be expanded.

B. Background and Phase I Technical Objectives

1. Background – Current manufacturing practices

a) Fiber Optic Interrogated Micro Well Sensors

Sensors created by etching microwells at the distal end of individual fibers within a fiber optic array were initially developed by Professor David Walt and colleagues at Tufts University. (reference 1). Fiber Optic Interrogated Microwell Plates are produced by fusing multiple optical fibers to form a fiber optic array. Selective removal of core material leaves wells that serve as massively parallel, minute 'test tubes'. 'Test tube bottoms' consist of high numerical aperture fiber optic cores that can be used to optically monitor reactions occurring in the wells, when coupled to a CCD. Well dimensions can be custom fabricated and range in diameter from 3μ - 250μ. In a typical application, 370,000 discrete reaction wells each 44μ in diameter populate a 25 X 75 mm slide. Larger slides 40 mm X 75 mm contain 1,400,000 discrete 44μ in diameter reaction wells. In other applications where smaller diameter wells are used, many millions of discrete reaction wells can be formed on a single slide.

Fiber Optic Interrogated Microwell Plates offer a variety of advantages as a platform for biosensors. The physical barrier provided by wells allows much higher sample density compared to alternative microarray techniques (ink jet, others) that depend on surface chemistry alone to separate one microdot from another. The high numerical aperture (NA) designed into the fiber optic core provides direct optical linkage to the reaction wells, which can then be interrogated by a variety of sensors, such as CCD. Use of EMA fibers eliminates optical cross talk from well to well

Fiber Optic Interrogated Microwell Plates are finding critical 'life sciences' applications as biosensors or 'biochips' as evidenced by the following titles from recent literature:

- "High-density fiber-optic DNA Random Microsphere Array" (reference 2)
- "High-density fiber optic array technology and its applications in functional genomic studies" (reference 3)
- "A massively parallel PitoTiterPlate based platform for discrete picoliter-scale polymerase chain reactions" (reference 4)
- "BeadArray Technology: Enabling an Accurate Cost-Effective Approach to High-Throughput Genotyping" [5]
- "RNA Profiling with Bead-Based Array Platforms" [6]

Varieties of analytical techniques have been developed to take advantage of the unique properties of fiber optic interrogated microwell structure. In a typical application, DNA fragments are isolated, bound to beads, and deposited into the minute wells of a fiber optic microarray. Reagents flow over the plate in a sequenced order. Light is released when a nucleotide binds with its complement on each DNA fragment. Each well is aligned with multiple pixels of a CCD camera, which capture the light signal. Massive data analysis is used to assemble the DNA sequence.

Other applications however involve loading the wells with living cells. Most cell types can be accommodated by matching fiber optic core size to the cell size requirements.[7] Specific diameter and depth dimensions of the well depend on the species of cell being studied, and might vary according to the specific technique being used to grow and analyze the cells. The typical range of well diameters that might be required range from 3 μ to 240 μ. While

technically feasible, this range of requirements creates special "inventory management" demands on the firms manufacturing fiber optic interrogated microwell plates , and has practical cost implications. Another technical disadvantage is that large wells interrogated by a single fiber, do not offer the same optical resolution as the same wells interrogated by many smaller fibers.

2. Manufacture of fiber optic arrays.

Incom Inc. of Charlton, MA is the leading manufacturer of fiber optic arrays. Fiber optic arrays are used in a variety of products such as:

- Faceplates: A faceplate is the optical equivalent of a zero thickness window providing a high dielectric strength vacuum interface that can also be used for field-flattening, distortion correction and contrast enhancement.
- Image Conduit: Image conduit is a high-quality image transfer device containing a rigidly fused bundle of optical fibers arranged in an ordered fashion to transmit an image from one end of the rigid fiber optic rod and display it at the other end of the rod.
- Tapers: Fiber optic tapers offer a low distortion method of magnifying or reducing an image, in image transfer applications.
- Inverters: Inverters are a special type of fiber optic plate that rotate an image through a predetermined angle, such as 180 degrees.
- Capillaries: Capillaries are a high-quality ordered array, similar to image conduit, except the solid optical fibers are replaced with hollow glass tubes.

Figure 1 and Figure 2 below show optical fiber faceplates and tapers:

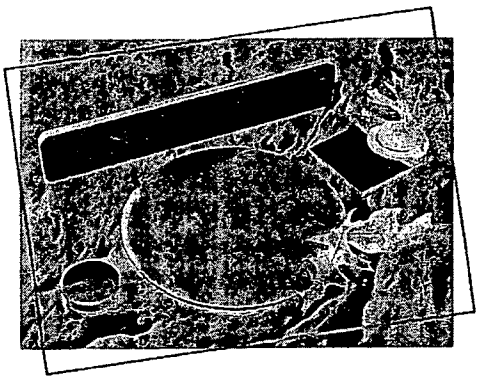

Figure 1. - Optical Faceplates

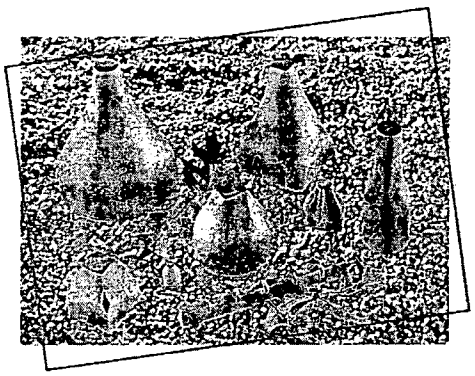

Figure 1 - Fiber Optic Tapers

These products are found in many commercial and military applications, such as cathode ray tubes, image intensifiers, and night vision systems.

The manufacturing practices for fabricating these and other fiber optic array products are well established. The starting point for manufacture is core glass rods, and clad glass tubing. The core glass is sized to fit closely within the clad tube. Together they are loaded into a draw furnace for the initial fabrication of cane. During initial 'cane draw', core glass and cladding

are fused together. Final cane diameter varies depending on the target dimensions (core diameter) for the finished product, but is typically about XYZ mm (Joe Please insert number) . Long lengths of cane are assembled into hexagonal close packed billets, which are re-drawn forming the first 'multi'. The process is repeated, with 'multi' assembled into a second billet, which is re-drawn to form 'multi-multi' cane. Incom proprietary processing yields the most blemish free, low distortion, zero-shear fused fiber optic components available. This fully developed manufacturing process, can now achieve multi/multi fibers up to 60mm diameter containing approximately 40 million fibers instead of the conventional thousands.

When multi-multi draw has been completed, fiber diameter has been reduced to the finished dimension, and no further reduction is required. During the "mold load" stage, "multi-multi's" are cut to the desired block length and stacked into a pressing fixture (typically about the size of a loaf of bread). Once assembled the mold is placed into a pressing furnace. During 'pressing', the furnace heats and softens the fiber array. A load is applied to ensure uniform fusion of the multi-multi's. Following fusion, the block is annealed and fabricated into finished product. In the case of fiber optic array plates, the block material is cut with wafer processing equipment into rectangular plates having the desired nominal thickness. The plates are then ground and polished to the target dimensions using conventional glass finishing slurry and pad materials. Figure 3 is a pictorial representation of the manufacturing process. Additional details are available at the Incom web site (www.incomusa.com).

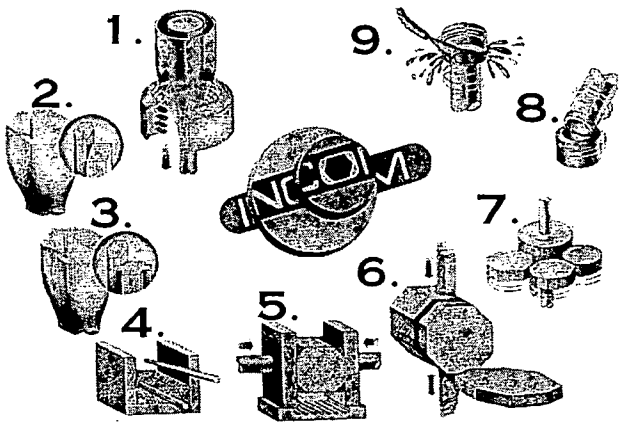

Figure 2– Incom, Inc. manufacturing process for fused fiber optic products, including fiber optic arrays.

An important consideration in the overall cost structure of fiber optic arrays is the cost of core glass rods. Core glass compositions are custom formulated and melted to meet unique optical requirements. In order to achieve the required quality, including optical homogeneity, melt 'campaigns' are conducted, typically involving approximately 100,000 lbs of glass, or more, at one time. In addition to raw material costs, the amortized cost of the glass must include significant furnace set-up fees, as well as the carrying costs for maintaining inventory over a number of years. The cost structure for producing fiber optic arrays greatly favors those compositions that are used in high volume.

Varieties of different clad glass tubing compositions are also available for use in producing fiber optic arrays. The clad glass must be properly selected to insure good chemical and mechanical match to the core glass. Many of the clad glass compositions that have proven to be useful over many years of experience are produced by the major glass manufacturers in very high volumes for other applications. These glass tubes can be purchased as needed and do not represent the same 'inventory' issue presented by custom melts of proprietary core glass compositions.

Glass cost is an important consideration in understanding the cost of fiber optic interrogated microwell plates . Certain compositions cost half or one third, compared to others, based on total volume used and the associated melting costs. These material cost differentials can have a significant impact on final product costs when yields and utilizations are considered in a complex multi step manufacturing process.

3. Etching fiber optic arrays to form Fiber Optic Interrogated MicroWell Sensors

Because of the unique position that Incom Inc. enjoys, as the premier supplier of fiber optic arrays for various applications, the company has also emerged a leading supplier of fiber optic interrogated micro well plates. Incom Inc. has established dedicated facilities for etching fiber optic arrays to form fiber optic interrogated microwell plates.

Fabricating Fiber Optic Interrogated Microwell Plates from fiber optic arrays takes advantage of the fact that the high refractive index glasses selected for the core material typically have much higher chemical solubility compared to the solubility of the clad glass. Indeed, the clad glass (tubing) selected is often a borosilicate composition highly regarded for its high chemical durability. The general process for selectively etching away core materials to form fiber optic interrogated micro wells has been described in the literature (reference #1). A variety of different mineral acids can be used depending on the specific composition of the glass cores being processed. Solution chemistry and process details are typically optimized to produce uniformly etched wells, with no residual layer of etch byproducts remaining on the micro well array. In some cases, it is necessary to use a multi-step 'etch' & 'clean' procedure to insure that the well surface quality meets requirements.

Before etching, fiber optic arrays are cut, ground, polished, and cleaned to meet the dimensional requirements of the biochip sensor. It is generally preferable to minimize handling, and to avoid any processing steps that have potential to contaminate the etched wells, once they are formed. Very high cleanliness standards must be preserved in order to meet the stringent requirements needed for genomic or proteomic analysis. In use, etched wells will be filled from the etched side, and optically interrogated from the opposite polished side. One option is to mask one side so that only one side is etched rather than etching both sides and re-polishing the other.

The etch process has proven to be highly reproducible when optimized using standard process controls. Finished fiber optic interrogated micro well plates must meet rigorous product specifications for dimensional tolerances, surface finish and quality, well diameter, depth and pitch (center to center distance between wells), as well as full product trace-ability. Fiber optic interrogated micro well plates are typically inspected visually and microscopically,

using an interference microscope to monitor well dimensions. Typical SEM and interference microscope images are shown in figure 4 and figure 5:

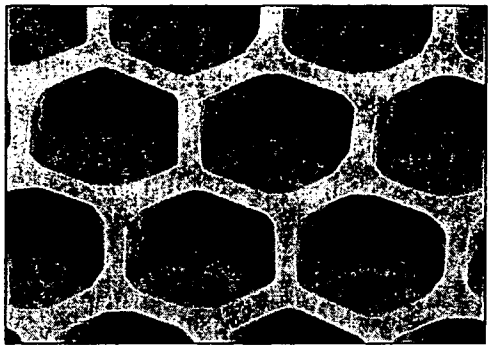

Figure 3 - SEM image showing fiber optic interrogated wells formed by etching a fiber optic array.

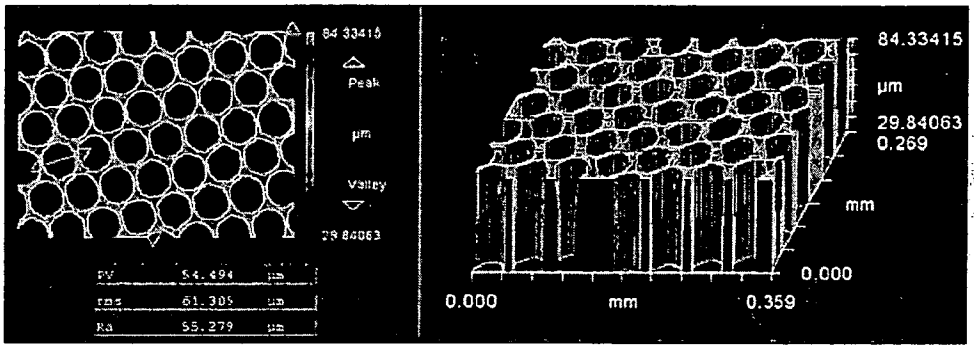

Figure 4 - Interference microscope image used to monitor the depth of fiber optic interrogated wells formed by etching a fiber optic array.

4. Limitations of Etch Processing to Fabricate Fiber Optic Interrogated Micro Well Sensors:

Etching is Limited to Certain Glass Compositions: The current etch process can only be advantageously applied to certain glass compositions that meet the optical requirements specified for this application, and which can also be economically etched to selectively remove core glass without removing clad glass. Compositions that meet the optical requirements and which might be advantageous from a cost perspective might not be able to be effectively etched. For example, not all glass compositions can be etched without leaving undesirable residual surface films. Furthermore, etching can distort the surface or well bottom morphology in ways that are not desirable or easily controlled. The limitations implied by the etch process are often only overcome by using glass compositions that are more expensive than others are.

Alternative processes that overcome these disadvantages by decoupling issues associated with glass composition with the well forming step would be highly desirable. This would also open the potential of creating micro fluid wells on the surface of fiber optic array plates made from glass compositions that are inherently lower cost compared to other, resulting in a considerable cost reduction.

Standardization of glass substrate: As previously discussed, well diameter, depth and pitch requirements for fiber interrogated micro well plates varies considerably depending on the specific samples being evaluated (genomic, proteomic, bacteria, mammalian cells, etc.), and the diagnostic techniques being used. While fiber optic arrays can be custom fabricated to meet these requirements, the lack of glass substrate standardization adds to the cost, and places a significant burden on maintaining glass inventories for each possible size.

Hazardous Materials & Waste Disposal: A practical consideration in the commercialization of this process is the cost associated with disposal of hazardous wastes. The mineral acids used to etch the glass require careful handling to insure the safety of personnel, and must be disposed of properly. The high refractive index core glasses specified for these applications include constituents such as PbO, BaO, La_2O_3, and Nb_2O_5. Costs associated with disposal of these hazardous wastes can add considerably to the final cost of the biochip product.

Optical Resolution: As previously explained, certain diagnostic applications require large well sizes. For example, wells with diameters of 100μ – 150μ or larger might be required for certain biological cells. Using the existing process, fiber optic arrays can be fabricated so that the core diameter of the fiber meets these dimensional requirements. This approach limits the resolution achieved since the entire contents of the well would be optically interrogated by a single fiber having a large diameter core.

It might be more advantageous to have a fabrication process that would make it possible to decouple well dimensions from the diameter of the fiber used in the fiber optic array. In this case, well diameter and depth could be optimized to the biological sample being tested, while the fiber diameter of the underlying substrate could be selected to optimize the dimensional requirements of optical detection system being used. For example, it might be desirable to create large diameter wells (100μ diameter or larger) onto a fiber optic array with 3μ diameter fibers. While not impossible, this design is not easily achieved using a manufacturing strategy that depends on the differential chemical solubility of clad glass vs. core glass.

Microchannel Structures: Differences in chemical solubility between core and clad glass provide an effective strategy to fabricate wells in the surface of fiber optic array plates. It is highly desirable to combine fiber interrogated well structures with other structures such as loading ports, mixing and reaction reservoirs, and flow channels to produce a fully integrated microfluidic, disposable microchip. Some authors report success fabricating glass microdevices using a fluorosilicic acid etching technique combined with photo-lithography and acid resists on homogeneous glass substrates[8]. There is no evidence however to suggest that these techniques could be successfully applied to a composite structure involving multiple glass compositions with different chemical solubility's, such as a fiber optic micro array plate

Labeling: It is highly desirable to be ably to apply unique labels to the fiber optic micro array plate facilitating traceability of samples. Ideally, the label would be microscopic, easily

read by the diagnostic instrument, permanent, and readily applied to the micro array plate without damaging or interfering with the surface of the micro array plate. Laser scribed bar codes microcrack and damage the surface of the glass plate and are not satisfactory. Traditional inks are not well adhered to glass. Ceramic frits applied to the glass surface create a raised surface profile that interferes with sealing the plate to prevent leaks. No labeling strategies have been demonstrated that can be applied in conjunction with the etched well manufacturing strategy.

Lean Manufacturing, Just-in-time delivery: The lead times to fabricate fiber optic array plates with custom fiber diameters are approximately 8 weeks. Manufacturing strategies that are not able to standardize on a small number of different fiber array substrates do not capitalize on the advantages of lean manufacturing.

5. Genomic Analysis using a Fiber Optic Interrogated Microwell Plate

The purpose of this section is to show how one firm is using Incom manufactured Fiber Optic Interrogated Microwell Plates for high speed genomic analysis. Another objective is to demonstrate that there are significant opportunities to integrate microfluidic design features into a low cost biochip that could be used directly without special loading fixtures or amplification chambers.

454 Corporation of Branford, Connecticut has been a pioneer in developing high speed, massively parallel analytical techniques based on the Fiber Optic Interrogated Micro Well Sensor. The diagram shown in Figure 6 depicts the analysis scheme developed by 454 Corporation[9]: 1) DNA fragments are isolated, amplified, bound to beads and 2) deposited into minute wells on a fiber optic microarray (PTP), 3) Reagents flow over the plate in a sequenced order. Light is released when a nucleotide binds with its complement on each DNA fragment. 4) Each well is aligned with multiple pixels of a CCD camera which capture the light signal which is gathered and stored with data acquisition equipment. 5) Massive data analysis is used to assembly the DNA sequence.

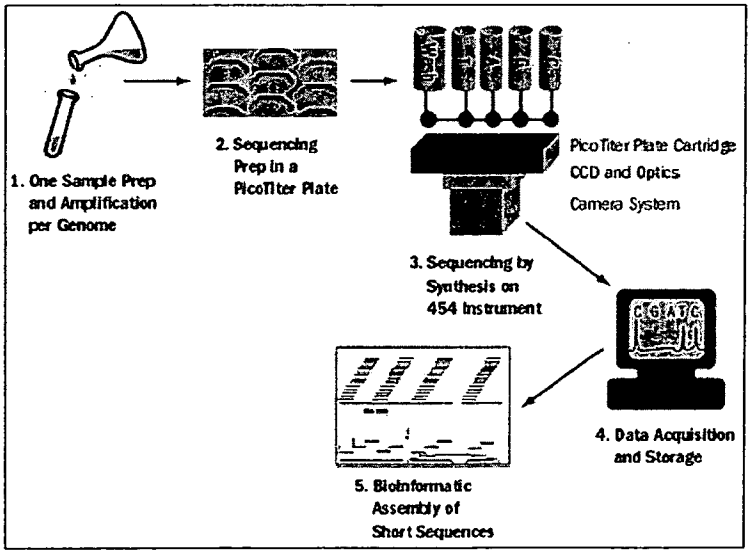

Figure 5 - Figure UVW: 454 C rporation Analysis Scheme

The elegance of the 454 analysis scheme lies is the fact that the underlying methodology is inherently simple! Despite this inherent simplicity, significant challenges have been addressed integrating the Fiber Optic Interrogated Microwell Plate with the CCD camera and the fluid flow systems, insuring uniform, controlled flow of reagents.

Figure 7 shows how the Fiber Optic Interrogated Microwell Plate loading cartridge employed by 454 Corporation[4]: (A) Fiber Optic Interrogated Micro Well Plate with microwells facing into the cartridge, the distance between the open sides of the Fiber Optic Interrogated Micro Well Plate wells and the wall of the loading cartridge is 0.3 mm; (B) silicon sealing gasket; (C) inlet port; (D) inlet loading tube; (E) outlet port; (F) outlet tube. The Fiber Optic Interrogated Micro Well Plate is held in the cartridge with plastic clamps (not shown). The liquid is filled via the inlet loading tube D and enters the space between the open sides of the Fiber Optic Interrogated Micro Well Plate wells and the wall of the loading cartridge through the inlet port C. The area defined by the silicon sealing gasket B is filled and excess liquid leaves the cartridge via the outlet port E and the outlet tubing F.

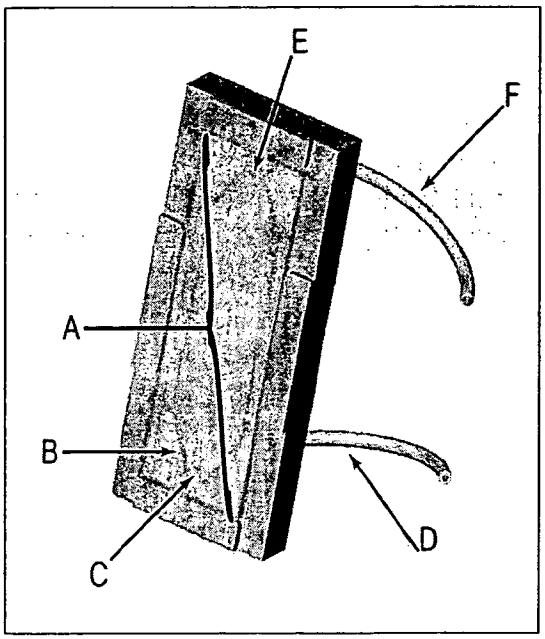

Figure 6 - Fiber Optic Interrogated Micro Well Plate loading cartridge.

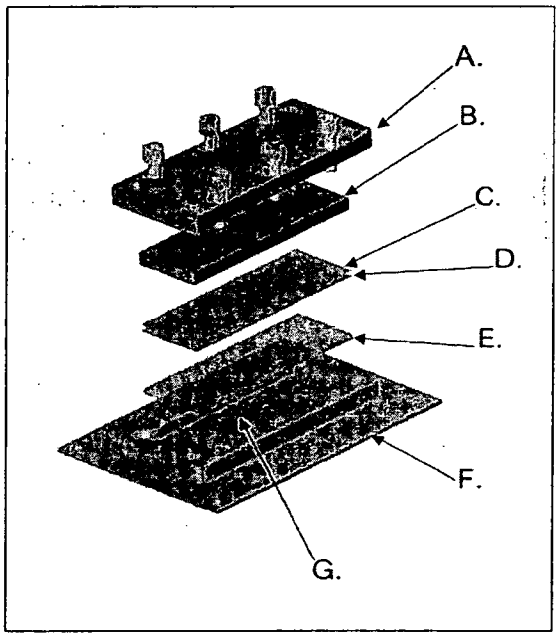

Figure 7 – Amplification Chamber

In a typical application the Fiber Optic Interrogated Micro Well Plate – Loading Cartridge Assembly is filled with PCR reaction mix, and allowed to incubate. After a brief period of time, the Fiber Optic Interrogated Microwell Plate is placed into the amplification chamber shown in Figure 8: (A) Amplification chamber lid with six retaining bolts, (B) closed cell foam insulation pad, (C) 25 X 75 mm standard glass microscope slide, (D) 0.25 mm thick silicon sheet, (E) Fiber Optic Interrogated Micro Well Plate, (F) amplification chamber base, (G) second 0.25 mm thick silicon sheet. The loading cartridge and amplification chamber add

time and complexity as well as potential for leaks, uneven reagent flow and cross contamination to the sample set-up process.

In the next section, an alternative strategy for fabrication of Fiber Optic Interrogated Microwell Plates is described. The proposed manufacturing strategy offers a number of benefits compared to manufacturing by chemical etching. A key feature of the proposed technique is the option to incorporate microfluidic design features that would eliminate the need for separate loading chambers or amplification chambers, in a low cost, disposable biochip.

6. An Alternative Strategy for Fabrication of Fiber Optic Interrogated Microwell Plate without Etching using Permanent Photo Curable Resists

The SU-8 is a negative, epoxy-type, UV sensitive photoresist that was first developed, and patented by IBM[10]. When coated on a substrate, and exposed to ultraviolet light (365 to 436 nm), the epoxy resist will form a highly structured cross-linked matrix with excellent adhesion properties. In addition, this resist system has three important attributes which make it very suitable for thick-film applications.

1. Having a low molecular weight, SU-8 can be readily dissolved in a variety of organic solvents.[1] .

2. SU-8 is characterized by a very low optical absorption in the UV range. As a consequence, these materials can be deposited and successfully cured in the form of thick films. Thicknesses of 200 μm can be obtained with a single spin-coating. Patterned coatings as thick as 2 mm and aspect ratio >20 have been demonstrated with a standard contact lithography equipment[11, 12] .

3. The exposed resist forms a highly cross-linked matrix that is thermally and chemically stable, making it more suitable for a variety of practical applications.

The proposed research is based on evaluating various formulations of SU-8 (including SU-8 2000-2025, and others) manufactured by MicroChem Corporation of Newton MA[13]. These materials are generally believed to have good adhesion properties to glass. This family of materials has also been shown to be amenable to multilayer coating techniques that enable formation of embedded micro-channels[14]. Structures having exceptionally high aspect ratios and straight sidewalls are readily formed in thick films by contact-proximity or projection printing. Cured SU-8 is highly resistant to solvents, acids and bases and has excellent thermal stability, making it well suited for applications in which cured structures are a permanent part of the device.

A principal goal of our development efforts are to apply permanent photocurable resist materials such as SU-8 to a fiber optic array substrates, forming fiber optic interrogated

[1] PGMEA (propylene glycol methyl ether acetate), GBL (gamma-butyrolactone), or MIBK (methyl iso-butyl ketone).

analysis wells while eliminating the need for etching. Furthermore, the techniques developed will demonstrate capability to produce fully integrated microfluidic devices that incorporate components such as loading ports, flow control channels, mixing and reaction zones, fiber optic interrogated analysis wells, nutrient channels, and recovery ports in a low cost biochip suitable for one time use. Ultimately, the objective of this research is to develop a low cost, disposable, high resolution biochip.

7. Application of SU-8 to Fiber Optic Arrays

A glass fiber optic array substrate will be fabricated in the shape of a circular disc to facilitate the application of the liquid resist using standard spin application techniques. All processing is done under yellow lights selected to prevent premature exposure of the photo resist. Detail processing steps included in the attached product specifications. The overview is as follows:

1. Substrate Pretreatment,
2. Coat,
3. Soft Bake,
4. Expose
5. Post Expose Bake
6. Develop
7. Rinse & Dry
8. Hard Bake / Cure

After exposure, the coating is selectively removed, or developed, to reveal the desired pattern of wells. The remaining coating material is then cured, to form a hard layer which becomes an intimate part of the final product. Once the hard bake / cure step has been completed, the cross-linked epoxy coating is extremely difficult to remove.

The first objective of this development program, formation of fiber optic interrogated wells on the surface of fiber optic array plates, will be achieved using the 'single layer' process described above. A first layer of SU-8 is deposited onto the substrate: following exposure, thermal curing and development, wells are formed. Optical fibers in the glass substrate forms the bottom of the wells.

The second objective of the program is to develop capability to form microfluidic structures such as loading ports, flow control channels, mixing and reaction zones, nutrient channels, or recovery ports formation. These structures require that a second layer must be deposited on top of the first, such that the top of the first layer becomes the bottom of second layer. Previously cited work (15) reviews technical options that facilitate multi-layer coating. Figure 8 shows four strategies for forming embedded micro-structural features such as channels: a) SU-8 plus filling material to form channels, b) metal mask protects first layer allowing formation of channels, c) laminating a top layer and d) partial exposure.

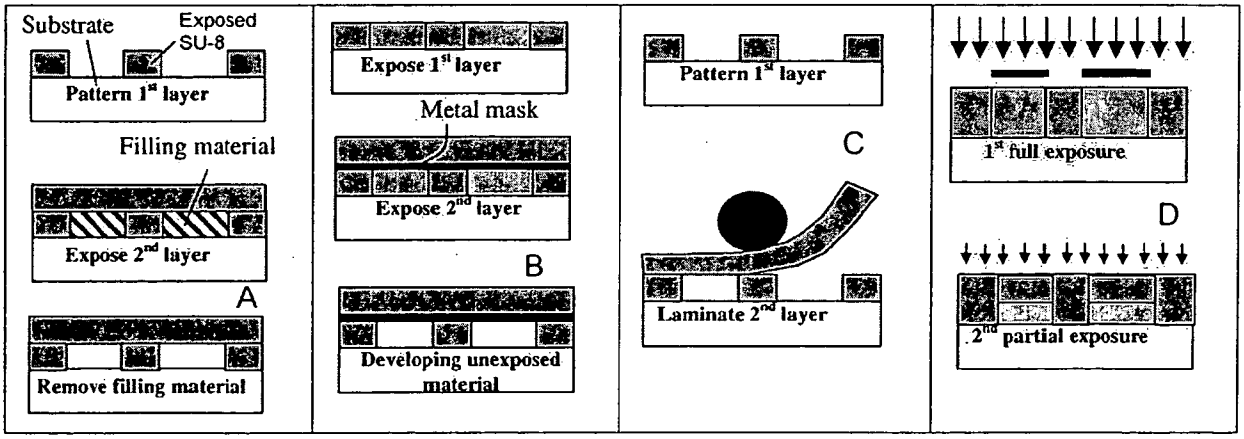

Figure 8 – Four methods for forming channels in SU-8

8. Advantages of the proposed technique

The techniques proposed to apply permanent photocurable resist materials such as SU-8 to a fiber optic array, forming fiber optic interrogated analysis wells have a number of distinct technical and commercial advantages over current etch techniques. These advantages are summarized in the following;

Applicable to All Glass Compositions: The old process could only be advantageously applied to certain glass compositions that had the optical properties that were desirable and which could be etched to selectively remove core glass without removing clad glass. The new process overcomes this disadvantage by decoupling issues associated with glass composition with the well forming step. This opens up the potential of creating micro fluid wells on the surface of fiber optic array plates made from glass compositions that are not suitable for etching or which are inherently lower cost compared to other compositions.

Cost Reduction: Standardization of glass substrate- The photo resist strategy for forming wells enables the manufacturer to 'standardize' the fiber optic array substrate, leading to significant cost reduction compared to etching, where a different substrate inventory is required for each well morphology. Another cost reduction is associated with eliminating the need for disposal of hazardous materials, which add considerably to the cost of the finished product.

Improved Resolution by Decoupling Fiber Size from Well Dimensions: The new process makes it possible to decouple well dimensions from the diameter of the fiber used in the fiber optic array. Well diameter and depth can be optimized to the biological sample being tested, while the fiber diameter of the underlying substrate can be selected to optimize the dimensional requirements of optical detection system being used. For example, it might be desirable to create large diameter wells (100μ diameter or larger) onto a fiber optic array with 3μ diameter fibers.

Integrated Microfluidic Structures - The proposed method of depositing a permanent epoxy based photo resist to form structures such as analysis wells on a fiber optic array plate can also be used to implement other more complex microfluidic structures, such as loading ports, mixing and reaction reservoirs, and flow channels. This can be used to enhance the ability to flow sequencing reagents uniformly across the entire surface of the plate which can have a significant impact on the sensitivity on the resolution and sensitivity of the diagnostic technique. In addition to improving flow uniformity, micro channels formed on the surface of the fiber optic faceplate can be used to separate the flow of reagents so that multiple sequencing reagents can be flowed across different parts of the biochip at the same time. For example, if the normal test calls for sequentially flowing 4 reagents, each separated by a rinsing step, the time required to complete the analysis can be significantly reduced by partitioning the biochip into distinct regions with its own micro channel flow, and flowing all 4 reagents across these different sections of the array, simultaneously.

Labeling: The uncured photo resist can be irradiated with a UV laser creating an alpha numeric or symbolic label which would be a permanent part of the finished product.

Lean, Just-in-time Manufacturing – The proposed process would enable rapid fabrication of biochips with custom microfluidic structures on a standardized fiber array substrate, facilitating low cost lean JIT manufacturing strategies.

9. Initial Trials & Preliminary Patent Application

Initial Trials were done on December 9, 2003, at MicroChem Corporation. A primary objective of these trials was to demonstrate the feasibility of forming fiber interrogated wells on micro array plates and identify issues that would need further resolution going forward. A second objective was to provide initial samples that would be helpful in engaging the interest of prospective customers. A third objective of these trials was to determine suitability for a preliminary patent application covering the concepts described in this proposal.

Substrate Materials: Incom Inc. provided two types of glass substrate for the trials:

- Window Glass – cut into 2 inch diameter discs ground and polished to a nominal 1 mm thickness. Trials using (low cost) window glass were included for initial trials learning the proper techniques to spin deposit the photo resist material to the appropriate thickness, as well as to find exposure levels that were appropriate for a non-reflective glass substrate.

- 3 micron Fiber Optic Faceplates – standard Incom faceplate material (X-5 Material: X14, C-5 core and clad glass compositions) fabricated into 2 inch discs ground and polished to a nominal 1 mm thickness. Detail specifications for the faceplate material are summarized in the following tables:

Substrate Surface Properties

Understanding and controlling the surface properties of the fiber optic array will likely play an important role in developing successful strategies to form multilayer microfluidic biosensors.

Optical interference measurements done on the micro array substrate plate confirm that even after polishing, the nominally 'flat' plate has considerable surface morphology.

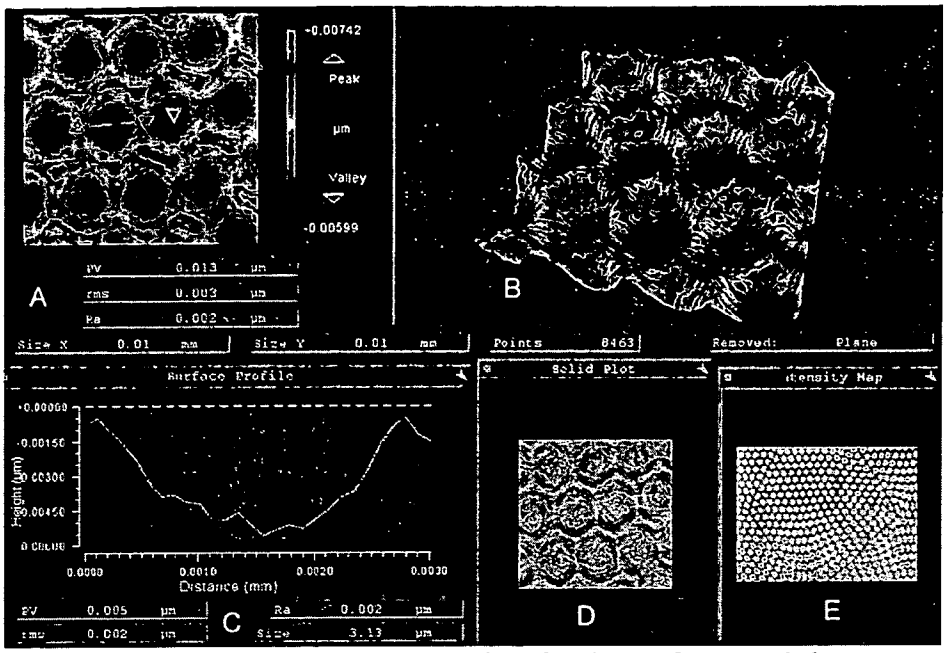

Figure 9 – Polished 3µ fiber optic array plate showing surface morphology. A) Filled Plot, B) 3D Model, C) Surface Profile, D) Solid Plot, F) Intensity Map.

The intensity map shown in figure 9 'E' shows individual 3µ fibers clustered in a 'multi'. During polishing, the softer core glass is removed faster than surrounding cladding, resulting in peak to valley differences of about 0.005µ, as shown in 'C'.

SU-8 Coating Trials: A 'chrome on fused silica' photo mask provided by MicroChem had a variety of test patterns including some that closely approximated the close packed well structures that were of interest for biochip application. Feasibility trials evaluated these variables:

1) Different photo resist materials: SU-8, SU-8 2000, SU-8 2025

2) Adhesion promoters intended to enhance the bond between substrate and photo resist: None, HMDS, XP Omni.

3) Substrate cleaning procedures.

4) Photo resist thickness (55 micron target)

5) UV exposure level: 100 mJ to 250 mJ

A series of feasibility trials were done using a photomask test pattern provided by MicroChem. Adequate control of coating thickness was achieved after the first few trials. Establishing good adhesion of the coating to the substrate proved to be more problematic. For some samples, entire sections of the test pattern delaminated from the substrate and were found floating in the developing solvents. Since these preliminary trials were not controlled experiments, drawing distinctions between the roles of exposure vs. substrate surface preparation vs. use of adhesion promoting agents could not be deduced.

The last two samples run used the 3 micron fiber array faceplate as substrate. Photo resist SU-8-2025 was applied at 1100 RPM. UV exposure for the last sample was increased to 250 mJ, based on the poor adhesion of earlier samples, the overall film thickness, and the fact that glass substrates often require more exposure since little UV is reflected back through the coating compared to silicon surfaces. Figure 10 below, is a low magnification image showing patterned SU-8 on a 3μ fiber array faceplate substrate, for the last sample run.

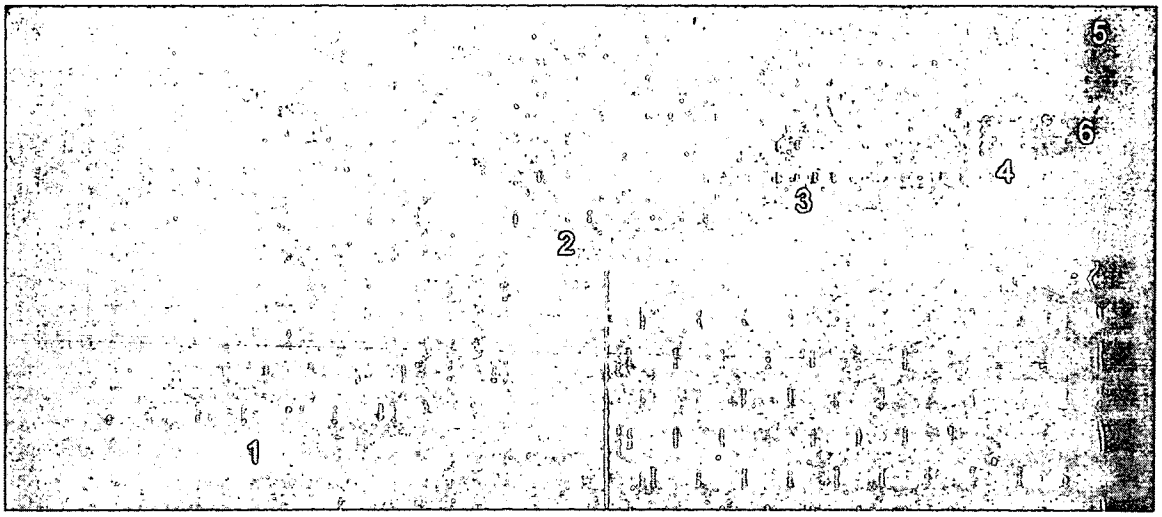

Figure 10 – A low magnification image showing patterned SU-8 on a 3μ fiber array faceplate substrate. Six different test patterns were deposited.

The dimensions of the different test patterns shown in Figure 11 are as follows:

#1 300μ diameter on 400μ pitch

#2 150μ diameter on 200μ pitch

#3 120μ diameter on 160μ pitch

#4 30μ diameter on 40μ pitch

#5 60μ diameter on 80μ pitch

#6 15μ diameter on 20μ pitch

These dimensions are based on the dimensions specified for the Chrome on Fused Silica mask. Direct measurement of the SU-8 test patterns will reveal whether any mask bias was experienced during deposition.

The photo resist test pattern on the last sample was sufficiently well adhered for initial samples. The film thickness was measured at 56-57 microns thick. Figures 11, 12 a dn 13 are photos were taken of pattern #3 (120μ cell, 160μ pitch) at higher magnification:

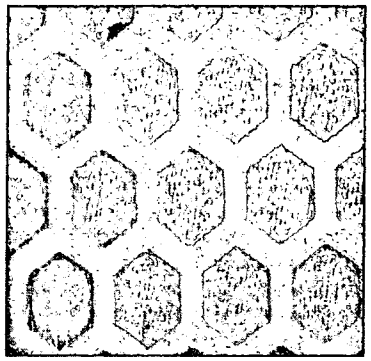

Figure 11 - Low magnification: focused onto the well bottom showing the 3μ diameter optical fiber structure.

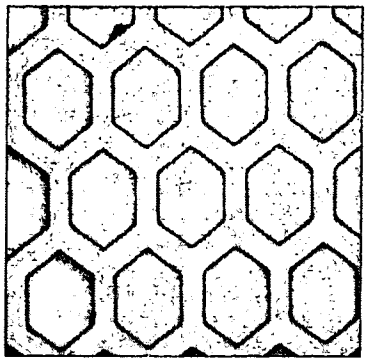

Figure 12 - Low magnification: focused onto the top surface of the SU-8 walls.

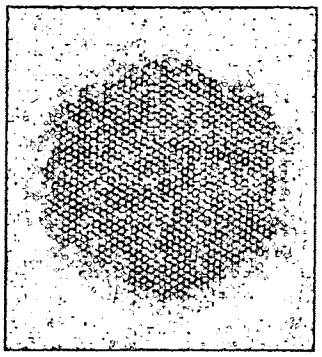

Figure 13 - High magnification: focused onto the well bottom showing the 3μ diameter optical fiber structure.

While demonstrating overall feasibility for the technique, the preliminary trials exposed a number of issues that will have to be resolved going forward, including:

Coating Adhesion – determining the roles played by exposure vs. substrate surface preparation vs. use of adhesion promoting agents could not be deduced.

Feature Resolution - No effort was made in the preliminary trials to optimize the feature resolution achieved. The image quality observed for features <30μ diameter indicate that further process development will be required to insure the quality required for certain microfluidic applications anticipated. We have good confidence in being able to achieve 5μ resolution, as evidenced by the photo shown in figure 14[15].

The feasibility trials also revealed other practical issues that will need to be resolved going forward. The 2" diameter substrates that were fabricated for these trials were too small compared to the 4" diameter photomask that was available. Difficulty was experienced insuring good alignment of the two.

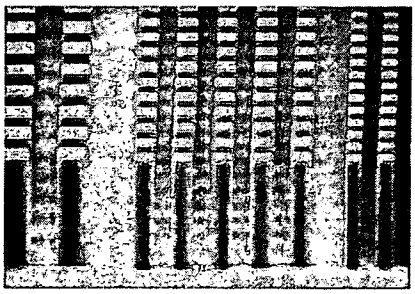

Figure 14 – 5μ, 10μ and 15μ post arrays formed with from a 50μ thick film f SU-8

10. Provisional Patent Application

The combination of SU-8 type photo curable resist materials applied to form microstructural features on a fiber optic array surface offers unique advantages compared to other techniques such as chemical etching. The success of these preliminary trials, and recognizing the potential for commercial development and application, has encouraged Incom Inc. to submit a provisional patent application covering this technology.

11. Phase I Technical Objectives

The first objectives of this research is to demonstrate that permanent photocurable resist materials such as SU-8 can be applied to a fiber optic array, forming fiber optic interrogated analysis wells, suitable for biosensor applications, while eliminating the need for etching. As part of this objective, the following will be accomplished:

- Explore processing conditions and demonstrate ability to fabricate micro arrays with a range over a full range of well dimensions: 6μ – 250 μ diameter, 6 μ - 55 μ deep
- Demonstrate that suitable adhesion is achieved to withstand exposure environments typical of biochip applications. For example, does the coating survive chemical exposure to PCR Mix over the time & temperature cycle typical for amplification?
- Determine whether the optical properties of the photocurable resist provide sufficient optical isolation between adjacent wells. Evaluate strategies to insure optical isolation such as absorbing colorants added to resist or surface coatings.
- Demonstrate that the photocurable resist does not have fluorescent characteristics that interfere with analysis.
- Evaluate the biocompatibility of the microarray.

A second objective of this research is to demonstrate capability to produce fully integrated microfluidic devices that incorporate components such as loading ports, flow control channels, mixing and reaction zones, fiber optic interrogated analysis wells, nutrient channels, and recovery ports in a low cost biochip suitable for one time use.

- Evaluate multilayer photo resist deposition masking, and developing techniques for forming flow channels leading into and out of fiber optic interrogated analysis wells.
- Evaluate fabrication techniques for forming a top cover & seal over fiber optic interrogated analysis wells, with capability to produce a fully embedded microfluidic device.

C. Phase I Research Plan

1. Substrate Preparation

Best practices procedures for applying permanent photocurable resist materials such as SU-8 onto a fiber optic array will be developed.

Glass Selection and Specification - A key objective of this development program is to demonstrate microwell formation on fiber optic array substrates fabricated from lower cost glass compositions. Two compositions will be evaluated as part of this effort.

X-5 fiber optic array materials are manufactured starting with X-14 core glass, and C-5 clad glass. This material lends itself well to etching, and is the preferred faceplate substrate used for FOI Micro Titer Plates manufactured by Incom using the traditional etching process. The feasibility trials described earlier were done using X-5 material

BPLI material, on the other hand, is made up of X-26 core glass and C-5 clad glass. This composition is optically equivalent to X-5 for biochip applications, and has the added advantage that X-26 is 2.5 times less expensive compared to X-14. This raw material savings translates into significant cost reduction in the finished part. Unfortunately, attempts to develop a etch process for X-26 have not been successful.

Technical specifications, including optical properties for both glass compositions is tabulated below. The technical plan going forward will evaluate SU-8 application to both substrate materials.

Table 1 - Core & Clad Glass Composition

Wt %	Clad C-5	Core X-14	Core X26
SiO_2	70-80	8-12	17-22
B_2O_3	5-10	8-12	4-6
Al_2O_3	5-10		5-7
K_2O	<5		
Na_2O	5-10		
BaO	<5	30-35	
CaO	<5		
PbO			35-45
Nb_2O_5		5-8	1-3
La_2O_3		18-25	14-17
ZnO			7-9
ZrO_2			1-3
As_2O_5			<5
TiO_2		12-16	
WO_2		< 5	

Table 2 - Glass properties

Property	Clad C-5	Core X-14	Core X26
Refractive Index	1.49	1.87	1.80
Density (g/cm^3)	2.36	4.54	4.81
CTE ($X10^{-7}$)	50	96	62

Anneal Pt. (C 10^{13})	580	650	555
Soft Pt. (C $10^{7.6}$)	798	734	687

Table 3 - Fiber Optic Array Faceplate Properties

Material Type	**BPLI-3, -6**	**X5**
Faceplate Sizes	up to 165mm	lg. diam & strip
Core Type	X26	X14
Clad Type	C5	C5
Core/Clad Ratio	12:1	14:1
EMA Type	B15	B15
EMA Design	Int	Int
MegaDraw	no	no
Fiber Size	3, or 6μ	3, or 6μ
N.A.	1.0	1.0
Density	3.90	3.84
Transmission	Figure 15	Figure 16
Collimated	79%	74%
Lambertian	56%	62%
Products	Inverters, Tapers, Faceplates	Faceplates

Material Type	BPLI-3, -6	X5
Core to Clad Ratio:	12:1	14:1
EMA Type:	B15	B15
EMA Design:	Interstitial	Interstitial
MEGAdraw:	no	no
Blockpress:	yes	yes
Numerical Aperture:	1.0	1.0
Density g/cm^3:	3.90	3.84
Thermal Expansion (0-300C)(x10^{-7}/C):	59.7	84.5
Thermal Conductivity: (across fiber end) (across length of fiber) W/m-K	 0.808 0.799	 0.834 0.758
Specific Heat: (across fiber end) (across length of fiber) J/g-K	 0.507 0.510	 0.532 0.502
Thermal Diffusivity (across fiber end) (across length of fiber) cm^2/s	 0.00403 0.00390	 0.00402 0.00387
Young's Modulus GPa:	78.04	93.11
Poisson's Ratio:	0.25	0.27
Shear Modulus GPa:	31.22	36.54

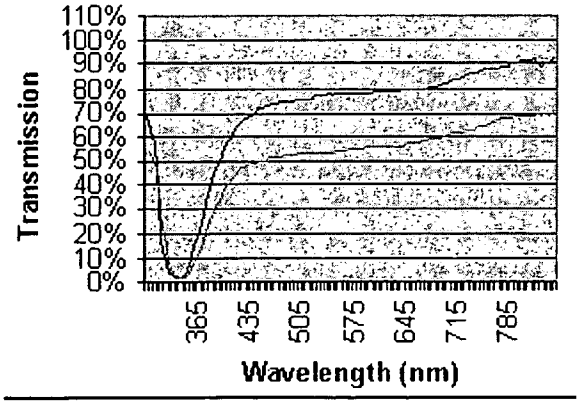

Figure 15 - BPLI lambertian & collimated transmission

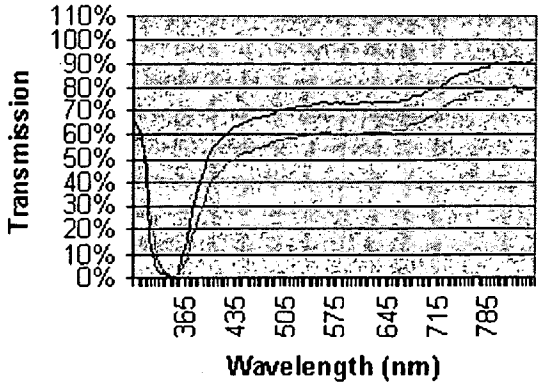

Figure 16 - X-5 lambertian & collimated transmission

Manufacture of substrate material - Incom Inc. will manufacture blocks of X-5 material and BPLI material for the program. The blocks will be sized sufficiently large to allow

fabrication of 4" diameter discs of glass. Blocks will be cut using an ID saw into wafers which will be ground to approximately 1 mm thickness. Wafers will be turned forming round discs. Each block is expected to produce approximately (JAK please estimate) 4" diameter X 1.0 mm thick discs.

The glass will be finished to standard specifications previously established for FOI Micro Titer Plate product produced by the standard etching technique. Incom QC personnel will inspect the discs monitoring the following parameters: Bevel, Blemish, Chicken Wire, Critical Zone, Dark Fibers, Dig, Edge Chips, Cracks & Fractures, Edge Marks / Clamp Damage, Fiber Size (Pitch), Flatness, Mechanical Dimensions, Polish, Scratch, Scratch / Dig.

Substrate Cleaning - Different techniques will be evaluated to determine the optimal surface cleaning procedures to insure good adhesion of SU-8 materials to the glass substrate. A proposed cleaning procedure will involve one or more of the following steps, alone or in combination: Contrad™ 70 detergent wash, DI rinse, Isopropyl Alcohol (IPA) rinse and drying, 400 C bake-out for 10 minutes. The initial "success criteria" for the cleaning will be evidence of low or zero water contact angle. Ultimately, the best measures of the cleaning procedure will be the impact on SU-8 adhesion. This will be evaluated by boiling coated substrates in water for 10, 20, or 30 minutes, and monitoring for evidence of coating detachment. Clean substrates will be stored in appropriately sized semiconductor wafer storage boxes.

Chrome on Glass Photomask - One or more photomasks will be required to support the proposed development efforts. The photomasks are intended to support exploration of the full range of features and resolution that might be of interest in FOI Micro Titer Plate biochips. Another objective is to insure that the samples produced are relevant to the test methods available to potential commercial customers. Final design criteria will be developed through discussions with current customers of the (etched) FOI Micro Titer Plates. Issues that will be addressed include a) well diameter (3-250μ), b) well spacing / pitch, c) well depth (3-55μ). In addition, options for microfluidic features such as loading ports, flow control channels, mixing and reaction zones, fiber optic interrogated analysis wells, nutrient channels, and recovery ports will also be discussed and included in the design.

Fabrication of the photomask will be done by an Advanced Reproductions Corporation.[2] The estimated cost for a photomask on soda lime glass, including 1-3 hours of design is $635 - $855. The breakdown is as follows: a) artwork @$110 per hour, b) 4" fabrication on soda lime glass @ $525, or $825 on fused quartz.

[2] Advance Reproductions Corporation, Precision Photomasks & Imaging Services, 100 Flagship Drive, North Andover, MA 01845

2. Coating Process Development

Photo resist material selection

SU-8 is a family of materials. A full series of products 'SU-8 2000 – 2025' is available optimized for different levels of coating thickness. Other versions are available which incorporate different solvents or which have been optimized for adhesion to different surfaces. Trials will be undertaken to select the best system for application to X-5 or BPLI glass surfaces.

Surface preparation

Pre-coating cleaning and/or bake out techniques might be required to insure optimal surface adhesion. Supplemental surface bonding agents are also available and will be evaluated.

Deposition techniques

Spin deposition techniques for applying photoresist are well documented. None-the-less, process trials will be needed to determine the optimal procedure for these coatings on the X-5 or BPLI substrates. Issues to be addressed include a) Spin speed, b) Ramp rates, c) Dwell times, d) Volume of photo resist applied. A key objective of these trials is to insure that target layer thickness is achieved, and that the coating is smooth and uniform. Following deposition, the coating is subjected to a 'soft bake' that insures that the coating is tack free so that the photomask can be applied without sticking.

UV Exposure

The UV exposure step, including the exposure time, intensity and wavelength (use of filters) has a significant impact on many variables that are important to the success of this effort. Coating adhesion and the resolution of features fabricated are directly affected by the exposure step.

Post Expose Bake

Time and temperature of this thermal treatment will be adjusted based on the thickness of the coating.

Develop

Alternative solvents and development times will be evaluated to optimize dissolution of unreacted SU-8.

Rinse & Dry

Samples will be rinsed in fresh developer to remove any residues, followed by IPA and DI water, followed by air drying.

Hard Bake / Cure

Time and temperature of this thermal treatment will be adjusted based on the thickness of the coating.

Accelerated Application & Analytical Testing

Accelerated applications testing will be done by exposing the coatings to boiling water and a PCR mix with repeat cycling to temperature. Coatings will be inspected for signs of deterioration.

Inspection techniques will include Optical Microscopic, which will be done at MicroChem Corporation as well as the University of Connecticut. Samples will also be submitted for SEM inspection, using facilities at University of Connecticut, as well as MAS, a commercial laboratory based in Raleigh, NC.

Best Practices Procedures Documented

Best practices procedures will be documented and will be followed during subsequent sample preparation.

3. FOI Micro Titer Plate Performance Testing

FOI Micro Titer Plate samples will be fabricated using the best practices coating procedures developed and will be subjected to a variety of performance tests by prospective users of the technology, including 454 Corporation (MAD?) and Dr. David Walt, at Tufts University (MJM?).

A number of key variables will be addressed through these tests. For example, it will be important to determine whether SU-8 provides adequate optical isolation between adjacent wells for fluorescence based analysis. If not, different strategies, such as the use of absorbing colorants, or surface coatings, will be considered. It will also be important to evaluate the biocompatibility of the SU-8 based sensor. The use of coatings such as a thin layer of SiO_2 could be used, if necessary.

Ultimate acceptance of FOI Micro Titer Plates made with SU-8 vs.etching will come through qualification trials intended to demonstrate that the two biochips provide equivalent technical performance in side by side comparison.

4. Microfluidic Structures

A second objective of this research is to demonstrate capability to produce fully integrated microfluidic devices that incorporate components such as loading ports, flow control channels, mixing and reaction zones, fiber optic interrogated analysis wells, nutrient channels, and recovery ports in a low cost biochip suitable for one time use.

The key technical challenge in meeting this goal is to demonstrate multilayer SU-8 coating techniques, forming flow channels leading into and out of fiber optic interrogated analysis wells. A first layer of coating will be deposited, exposed, and baked, but not developed, using the 'best practices' techniques developed in the first part of the program. A second layer of SU-8 will be deposited over the first. A second photomask will be used to pattern channels that flow into the latent wells of the first layer. Finally, the two layer structure will be 'developed' using appropriate solvent to dissolve SU-8 from the channels leading to wells, and the wells themselves. Depending on the success of these trials, fabrication techniques for

forming a top cover & seal over fiber optic interrogated analysis wells, with capability to produce a fully embedded microfluidic device, will also be explored.

All samples will be subjected to the same accelerated application testing and FOI Micro Titer Plate testing as the single layer samples.

D. Company Information (JAK)

E. Commercial Potential (JAK)

F. Consultants and Subawards / Subcontracts

G. Equivalent or Overlapping Proposals

H. Letters of Support or Commitment

V. References Cited (see list at end of document)

VI. Biographical Sketches

A. Tony Detarando

B. Michael Detarando

C. Joseph Krans

D. Jason kass

E. Michael Minot (See Attachment)

F. David Stowe

VII. Budget

VIII. Current and Pending Support of PI and Senior Personnel

IX. Equipment, Instrumentation, Computers and Facilities

X. Supplementary Docs.

A. Company commercialization History

XI. List of Suggested Reviewers (??) get suggestions from 454, David Walt, etc.

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Jan Berka
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A massively parallel PicoTiterPlate™ based platform for discrete picoliter-scale polymerase chain reactions

We demonstrate successful, simultaneous polymerase chain reaction (PCR) amplification of up to 300 000 discrete reactions in a novel platform, the PicoTiterPlate™. In addition to elevated throughput, the PicoTiterPlate™ based amplifications (PTPCR) can be performed in extremely small volumes: individual reactions volumes are as low as 39.5 pL, with a total 15.3 μL reaction volume for the entire PicoTiterPlate™. The bulk PTPCR product can be recovered and assayed with real-time PCR, or discrete PTPCR products can be driven to solid supports, enabling downstream applications such as translation/transcription or sequencing.

Keywords: Amplification / Massively parallel / Picoliter / Polymerase chain reaction
DOI 10.1002/elps.200305646

1 Introduction

Over the last decade, the compilation of large volumes of genetic data has transcended academic interest and has become a vital component of many diverse fields, ranging from human health to agriculture and national defense. In most cases, polymerase chain reaction (PCR) [1, 2] plays an integral part in obtaining this information, amplifying minute amounts of specific DNA to sequenceable concentrations. Despite its ubiquitous presence and use in laboratories, scaling current PCR technology to meet the increasing demands of modern genetics is neither cost effective nor efficient, especially when the requirements for full genome sequencing are considered.

Efforts to maximize time and cost efficiencies have typically focused on two areas: decreasing the reaction volume required for amplifications and increasing the number of simultaneous amplifications performed. Miniaturization confers the advantage of lowered sample and reagent utilization, decreased amplification times and increased throughput scalability. While conventional thermocyclers require relatively long cycling times due to thermal mass restrictions [3], smaller reaction volumes can be cycled more rapidly. Continuous-flow PCR devices have utilized etched microchannels in conjunction with fixed temperature zones to reduce reaction volumes to sub-microliter sample levels [4, 5]. Glass microcapillaries, heated by air [6] or infrared light [7, 8], have also served as efficient vessels for nanoliter scale reactions. Similar reaction volumes have been attained with microfabricated silicon thermocyclers [9]. In many cases, these miniaturizations have reduced total PCR reaction times to less than 30 min for modified electric heating elements [10, 11] and hot air cyclers [6], and to 240 s for some infrared-controlled reactions [12].

Many technologies have addressed both throughput and miniaturization simultaneously, as in the 1536-well system design by Sasaki *et al.* [13] that maintained reaction volumes under 1 μL. Until now, one of the most dramatic combination of sample reduction and parallel amplification was achieved when Nagai *et al.* [14, 15] amplified a single test fragment in ten thousand 86 pL reaction pits etched in a single silicon wafer. Unfortunately, recovery and utilization of the amplicon have proven problematic, requiring evaporation through selectively permeable membranes.

Despite these remarkable improvements in reactions volumes and cycle times, none of these strategies provide the massively parallel amplification required to dramatically increase throughput to levels required for analysis of the entire human genome. Here, we report a novel platform, the PicoTiterPlate™, which permits simultaneous amplification of 300 000 discrete PCR reactions (PTPCR) in volumes as low as 39.5 pL. The pooled PTPCR products from the entire reaction can be recovered through a wash step and assayed *via* real-time PCR for the presence and abundance of specific templates. Of greater interest, we demonstrate that the PTPCR product

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Abbreviations: PTPCR, PicoTiterPlate™ polymerase chain reaction; **TaqMan®**, a registered trademark of Roche Molecular Systems for real time sequence detection; **TE**, Tris-EDTA buffer

can be driven to solid supports and detected by hybridization with two-color fluorescent probes. With this successful high-capacity, solid-phase, clonal DNA amplification it is not unreasonable to suppose that wider capabilities for this platform could be realized. The literature is replete with creative uses for solid-phase DNA, [16–22], *e.g.*, amplified DNA capture beads could be used as templates for sequencing reactions [23–25]. The large-scale parallel sequencing of thousands of clonally amplified targets should greatly facilitate large-scale, whole-genome library analysis without the time-consuming sample preparation process and expensive, error-prone cloning processes.

2 Materials and methods

Unless otherwise indicated, all common laboratory chemicals were purchased either from Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

2.1 PicoTiterPlates™

The PicoTiterPlates™ (25 × 75 × 2 mm) were manufactured by anisotropic etching of fiber optic face plates in a manner similar to that described in [26]. Plates were etched in three different microwell depths, 26, 50, and 76 μm. Microwell center-to-center pitch was 50 μm, and well diameters ranged between 39 and 44 μm (see Fig. 1), with a calculated well density of 480 wells/mm^2.

2.2 Solid-phase immobilization of oligonucleotide primers

Packed beads from a 1 mL *N*-hydroxysuccinimide ester (NHS)-activated Sepharose HP affinity column (Amersham Biosciences, Piscataway, NJ) were removed from the column and activated as described in the product literature (Amersham Pharmacia Protocol # 71700600AP). Twenty-five microliters of a 1 mM amine-labeled HEG capture primer (5'-Amine-3 hexaethyleneglycol spacers CCATCTGTTGCGTGCGTGTC-3') (IDT Technologies, Coralville, IA, USA) in 20 mM phosphate buffer, pH 8.0, were bound to the beads, after which 36–25 μm beads were selected by serial passage through 36 and 25 μm pore filter mesh sections (Sefar America, Depew, NY, USA). DNA capture beads that passed through the first filter, but were retained by the second were collected in bead storage buffer (50 mM Tris, 0.02% Tween, 0.02% sodium azide, pH 8), quantitated with a hemacytometer (Hausser Scientific, Horsham, PA, USA) and stored at 4°C until needed.

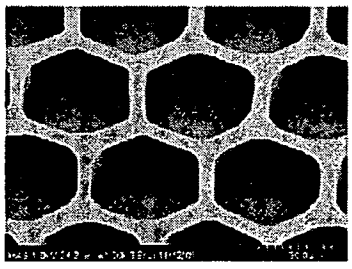

Figure 1. Scanning electron micrograph of a portion of a 25 × 75 mm PicoTiterPlate™ surface.

2.3 Generation of test DNA fragments

Amplification test fragments were derived from a commercially available adenovirus serotype 5 vector, pAdEasy (Stratagene, La Jolla, CA, USA). Fragments were amplified using bipartite PCR primers, the 5'-end of which contained a 20 base amplification region, and a 20 base 3'-section, complementary to a specific region of the adenovirus genome. Using these primers, two fragments were amplified from the 12933–13070 and 5659–5767 position of the adenovirus genome and assigned labels Fragment A and Fragment B, respectively. The sequence for the forward and reverse primers for Fragment A follow, with a slash (/) denoting the separation between the two regions of the primer: forward (5'-CGTTTCCCCTGTG TGCCTTG/CATCTTGTCCACTAGGCTCT-3'), reverse (5'-CCATCTGTTGCGTGCGTGTC/ACCAGCACTCGCACCA CC-3'). The primers for the Fragment B are as follows: forward (5'-CGTTTCCCCTGTGTGCCTTG/TACCTCTCC GCGTAGGCG-3'), and reverse (5'-CCATCTGTTGCGTG CGTGTC/CCCCGGACGAGACGCAG-3'). Reaction conditions were 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 μM each forward and reverse primer, 0.1 U/μL *Taq* (Promega, Madison, WI, USA) and 50 nmol template DNA. Both templates were amplified with a PCR program that consisted of 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s. Including PCR primers, the total length of the fragments was 178 bp for Fragment A and 148 bp for Fragment B.

2.4 Generation of fluorescent probes

Biotinylated double-stranded fluorescent probes were prepared by PCR amplification from the pAdEasy vector as described above, except that the primer sequences were changed to prevent hybridization between the test fragment and probe primer regions. In addition, the reverse primers for both fragments utilized a 5'-biotin fol-

lowed by 3 × hexaethyleneglycol spacers to permit product immobilization to beads prior to elution of the single-stranded probe. The sequence for the forward primer for the fluorescent Fragment A probe follows, with a slash (/) denoting the separation between the two regions of the primer (5'-ATCTCTGCCTACTAACCATGAAG/CATCTTGT CCACTAGGCTCT-3'), and the sequence for the reverse primer was 5'-biotin – 3 × hexaethyleneglycol spacers – GTTTCTCTCCAGCCTCTCACCGA/ACCAGCACTCGCA CCACC-3'. The primers for the Fragment B are as follows: forward (5'-ATCTCTGCCTACTAACCATGAAG/TACCTCT CCGCGTAGGCG-3'), reverse (5'-biotin – 3 × hexaethyleneglycol spacers – GTTTCTCTCCAGCCTCTCACCGA/ CCCCGGACGAGACGCAG-3'). Fluorescent moieties were incorporated through the nucleotide mix, which was comprised of 0.2 mM dATP/dGTP/dCTP, 0.15 mM TTP and 0.05 mM Alexa Fluor 488 – dUTP (Molecular Probes, Eugene, OR, USA) for Fragment A or 0.2 mM dATP/dGTP/ TTP, 0.15 mM dCTP, and 0.05 mM Alexa Fluor 647 – dCTP for amplifying Fragment B)(Molecular Probes). The fluorescent products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), and the biotinylated DNA was subsequently bound to 100 µL (approximately 1 million) Streptavidin Sepharose High Performance beads (Amersham Biosciences) in 1 × binding wash (5 mM Tris HCl, pH 7.5, 1 M NaCl, 0.5 mM EDTA, 0.05% Tween 20) for 2 h at room temperature. After incubation, the beads were washed three times in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated with 250 µL melt solution (0.125 N NaOH/0.1 M NaCl) for 2 min, releasing the single-stranded probe from the beads. Beads were pelleted with brief centrifugation in a benchtop centrifuge and the supernatant was neutralized in 1.25 mL buffer PB (Qiagen) with 1.9 µL glacial acetic acid. This mixture was repurified on a QiaQuick column (Qiagen), and the concentration of the purified probe was determined by TaqMan quantification using the BioRad iCycler (BioRad, Hercules, CA, USA).

2.5 Solution-phase PTPCR

To load PCR reaction mix into individual wells of a single 14 mm × 43 mm PicoTiterPlate™, 500 µL of PCR reaction mix (1 × Platinum HiFi Buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgSO_4$, 0.5% BSA, 1 mM dNTPs (MBI Fermentas, Hanover, MD, USA), 1 µM forward (5'-CGTT TCCCCTGTGTGCCTTG-3') and reverse (5'-CCATCTGT TGCGTGCGTGTC-3') primers, 0.05% Tween 80, 1 U/µL Platinum High Fidelity DNA Polymerase (Invitrogen), 0.003 U/µL thermostable pyrophosphatase (USB, Cleveland, OH, USA), and a calculated 5 copies of Fragment B template per well) were combined in a 1.5 mL microcentrifuge tube. The tube was vortexed thoroughly and stored on ice until the PicoTiterPlate™ loading cartridge was assembled. The in-house made PicoTiterPlate™ loading cartridge was attached to the PicoTiterPlate™ with two plastic clips, seating the silicon cartridge gasket firmly on the PicoTiterPlate™ surface (see Fig. 2). The PCR reaction mix was drawn into a 1 mL disposable syringe, and the mouth of the syringe inserted into the input tube of the loading cartridge. The loading cartridge was placed on end, so that the input port was oriented at the bottom of cartridge, and the PCR mix was slowly loaded into the chamber; even, bubble-free loading was ensured by inspection through the transparent back of the PicoTiterPlate™. After loading, the PCR mix was allowed to incubate for 5 min, at which time the reaction mix was withdrawn from the PicoTiterPlate™ loading cartridge.

The PicoTiterPlate™ was removed from the loading cartridge, and immediately placed in the amplification chamber (see Fig. 3). The PicoTiterPlate™ surface was covered with a 0.25 mm thick Silpad A-2000 silicon sheet (The Bergquist Company, Chanhassen, MN, USA), on top of which was placed a 25 mm × 75 mm standard glass microscope slide (Fisher), and a closed cell foam insulation pad (Wicks Aircraft Supply, Highland, IL, USA) was placed on top of the microscope slide. An aluminum lid attached to the base of the chamber by six 25 mm bolts

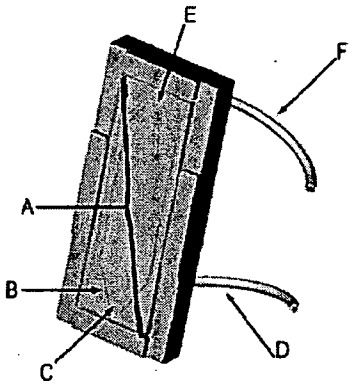

Figure 2. PicoTiterPlate™ loading cartridge. (A) PicoTiterPlate™ with microwells facing into the cartridge, the distance between the open sides of the PicoTiterPlate™ wells and the wall of the loading cartridge is 0.3 mm; (B) silicon sealing gasket; (C) inlet port; (D) inlet loading tube; (E) outlet port; (F) outlet tube. The PicoTiterPlate™ is held in the cartridge with plastic clamps (not shown). The liquid is filled via the inlet loading tube D and enters the space between the open sides of the PicoTiterPlate™ wells and the wall of the loading cartridge through the inlet port C. The area defined by the silicon sealing gasket B is filled and excess liquid leaves the cartridge via the outlet port E and the outlet tubing F.

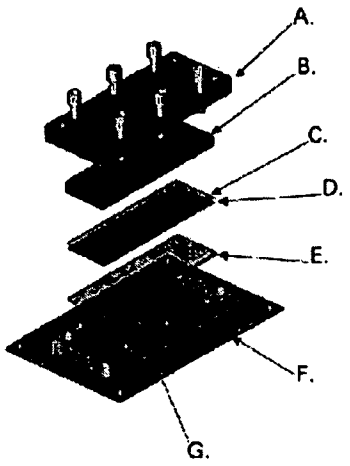

Figure 3. PicoTiterPlate™ Amplification chamber, exploded view. (A) Amplification chamber lid with six retaining bolts, (B) closed cell foam insulation pad, (C) 25 × 75 mm standard glass microscope slide, (D) 0.25 mm thick silicon sheet, (E) PicoTiterPlate™, (F) amplification chamber base, (G) second 0.25 mm thick silicon sheet.

sealed the amplification chamber. Once sealed, the amplification chamber was placed on a Thermocycler MJ PTC 225 Tetrad (MJ Research, Waltham, MA, USA) equipped with Flat Block Alpha Units, and an amplification program was run consisting of 3 min at 94°C (Hotstart Initiation), followed by 40 cycles of 12 s at 94°C, 12 s at 58°C, 12 s at 68°C with a 10°C final hold. After completion of the PCR program, the PicoTiterPlate™ was removed from the amplification chamber, and the loading cartridge was reattached. A disposable syringe was used to fill the cartridge chamber with 1 mL of H_2O, and allowed to incubate for 20 min at room temperature. After the incubation was completed, the recovery solution was withdrawn from the loading cartridge and transferred to a 1.5 mL microcentrifuge tube. PCR product was quantitated using an iCycler RealTime PCR unit (Bio-Rad) and carboxyfluorescein (FAM)-labeled reporter probes (Epoch Biosciences, Bothell, WA, USA). The TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City, CA, USA) was combined with 0.3 μM forward and reverse primers, 0.15 μM FAM-labeled probe, and 27 μL of the reaction mix added to each well in a 96-well PCR plate. Purified fragments were used to create a standard curve (six standards ranging from 1×10^9 to 1×10^4 molecules per well), which was run in triplicate. The PCR amplification was run with the following parameters: 5 min at 94°C (hotstart initiation), 60 cycles of 15 s at 94°C, 45 s at 68°C, with a final hold at 4°C. Data was analyzed using the iCycler Optical Systems Software Version 2.3 (Bio-Rad), and the PCR yield was quantitated using the iCycler data and Microsoft Excel (Microsoft, Redmond, WA, USA).

2.6 Solid-phase PCR

Solid-phase PTPCR was performed similarly to solution phase PTPCR, except that DNA capture beads were loaded into the PicoTiterPlate™ wells prior to amplification by centrifugation as described below, and the PCR mix was loaded into the microwells after the bead deposition was completed. To facilitate retention of the capture beads during wash steps, the solid phase experiments utilized 50 μm deep PicoTiterPlate™s. The PicoTiterPlate™ was placed in an in-house built plexiglass bead loading jig (similar to the PicoTiterPlate™ loading jig described in Fig. 2 except that a PicoTiterPlate™ was sandwiched between a bottom Plexiglas plate and a jig top plate, containing inlet and outlet ports, and sealed *via* a silicon gasket with plastic screws). Template DNA was preannealed to the DNA capture beads at 5 template copies per bead by incubation at 80 for 3 min, after which beads were allowed to cool to room temperature for 15 min. The beads were then spun into the PicoTiterPlate™ wells prior to loading the PCR reaction mix. Bead loading buffer (450 μL; 1 × Platinum HiFi PCR buffer (Invitrogen), 0.02% Tween 80) containing 100 000 Sepharose DNA capture beads (approximately 1 bead per 3 PicoTiterPlate™ wells) were injected by pipette into the jig through one of the inlet ports, and each inlet hole then sealed with a circular adhesive pad (3M VHS, St. Paul, MN, USA). The jig containing the PicoTiterPlate™ with its wells facing up and covered with the bead suspension were then centrifuged at 2000 rpm for 5 min at room temperature in an Allegra 6 centrifuge (Beckman Coulter, Fullerton, CA, USA) using a Microtiter Rotor. After centrifugation, the PicoTiterPlate™ was removed from the jig. The PCR reaction mix was loaded onto the PicoTiterPlate™ as described for solution-phase PCR, although as the template was preannealed to the DNA capture beads, the solid-phase PCR mix contained no template. The solid-phase PCR amplification program included additional hybridization/extension cycles to compensate for the immobilized primer's slower kinetics. The program comprised 3 min at 94°C for hotstart initiation, 40 cycles of 12 s at 94°C, 12 s at 58°C, 12 s at 68°C, followed by 10 cycles of 12 s at 94°C, 10 min at 68°C for hybridization, and extension with a 10°C final hold. Upon completion of the PCR program, the PicoTiterPlate™ was removed from the amplification chamber, and washed with 1 mL H_2O as described for solution-phase PCR. The PicoTiterPlate™ was then prepared for hybridization detection of immobilized PCR product.

2.7 Hybridization with fluorescently labeled probes

After PTPCR was complete, the strand complementary to the immobilized strand was removed by incubation of the whole PicoTiterPlate™ in 0.125 M NaOH for 8 min at room temperature, followed by neutralization in two 5 min washes in 50 mL of 20 mM Tris-acetate, pH 7.5. The PicoTiterPlate™ was then placed in a custom-made 800 µL hybridization chamber, and blocked with hybridization buffer (3.5 × saline sodium citrate (SSC), 3.0% SDS; 20 × SSC buffer is 3 M NaCl, 0.3 M Na_3-citrate) at 65°C for 30 min. The contents of the chamber were replaced with fresh hybridization buffer containing 20 nM fluorescent Fragment A (Alexa-488) and Fragment B (Alexa-647) probes. The probes were allowed to hybridize to their targets at 65°C for 4 h while shaking at 200 rpm on an orbital shaker (Barnstead International, Dubuque, IA, USA). After hybridization, the PicoTiterPlate™ was washed with 2 × SSC, 0.1% SDS for 15 min at 37°C, followed by a 15 min wash in 1 × SSC at 37°C, with two final 15 min washes in 0.2 × SSC at 37°C. Following post-hybridization washing, the PicoTiterPlate™s were air-dried and placed in a FLA-8000 Fluorescent Image Analyzer (Fujifilm Medical Systems USA, Stamford, CT, USA) and scanned at the 635 and 473 nm wavelength. The resulting 16-bit tiff images were imported into Genepix 4.0 (Axon Instruments, Union City, CA, USA), where a block of 100 analysis features was drawn over the area of interest and the 635 and 473 fluorescence intensities were recorded for each feature. Data was then exported to Microsoft Excel for further analysis.

2.8 Preparation of control beads

Biotinylated test templates A and B were prepared by PCR amplification from the pAdEasy vector, purified, immobilized on Streptavidin Sepharose High Performance beads and strand separated as described under Section 2.4, except no fluorescently labeled dNTPs were used in the PCR reaction. Pelleted beads were washed 3 times with TE buffer and stored at 4°C in TE until deposition onto the PicoTiterPlate™.

3 Results

3.1 Solution-phase PTPCR

Solution-phase amplification was demonstrated by loading PicoTiterPlate™s with PCR master mix containing a calculated 5 template copies per PicoTiterPlate™ well. Reactions were run in duplicate in PicoTiterPlate™s with 26, 50, and 76 µm deep wells. Forty cycles of PTPCR amplification were performed as described in Section 2.3. Additives were required to prevent the deleterious surface effects routinely reported with silica reaction vessels [6, 27, 28]. The inclusion of 0.5% BSA and 0.05% Tween 80 in the reaction mix was not only effective at reducing surface effects, but essential to amplification; reducing the relative concentrations of either reagent prohibited amplification entirely (data not shown). In addition, due to the polymerase-inactivating properties of silica surfaces [28, 29], elevated *Taq* concentrations proved beneficial. Concentrations above 1 U/µL showed little improvement in yield, but decreased polymerase concentrations exhibited reduced amplicon yield (data not shown).

Following PTPCR, the solution from each PicoTiterPlate™ was recovered and triplicate samples of each solution were quantified by TaqMan assay. A standard curve of diluted template (linear from 1×10^9 to 10^4 molecules, $r^2 = 0.995$) was used to determine the concentration of the amplified product. The number of molecules amplified per well was obtained by dividing the amount of amplified product by the total number of wells in a PicoTiterPlate™ (372 380). Fold amplification per well was calculated by dividing this number by the initial template concentration per well. PTPCR amplification was successful in all of the PicoTiterPlates™, with yields ranging from 2.36×10^6 fold in the 39.5 pl wells to 1.28×10^9 fold in the 50 pl wells (Table 1). Yield was influenced by well volume; the concentration of final product obtained for the 50 µm deep wells (1.4×10^{-4} M) was significantly greater (p-value for analysis of variance (ANOVA) = 0.023) than that obtained in the 76 µm (6.54×10^{-5} M) deep wells, both were two orders of magnitude greater than the yield achieved in the 26 µm deep wells (4.96×10^{-7} M). It may be that the 50 µm deep microwell yield represents the optimal balancing of the costs and benefits associated with low-volume PCR. In this case, maximum elevation of the effective concentrations and low thermal mass of the reagents are obtained, but the surface to volume ratio is still low enough to prevent detrimental surface effects from significantly reducing amplification efficiency.

Table 1. PicoTiterPlate™ PCR amplification as determined by TaqMan Assay

PicoTiterPlate™ depth (µm)	Well volume (pL)	Average fold amplification ($N = 6$)	Fold amplification (SD)	Final product concentration (M)
26	39.5	2.36E+06	1.02E+06	4.96E-07
50	76.0	1.28E+09	1.03E+09	1.40E-04
76	115.6	9.10E+08	4.95E+08	6.54E-05

Values reflect triplicate measurements taken from duplicate PicoTiterPlates™ ($N = 6$); SD, standard deviation

The final concentration of PTPCR product obtained in each of the different well depths (4.96×10^{-7} to 1.4×10^{-4} M) exceeds the 10^{-8} M concentration typically reported as the maximum achievable before the PCR plateau effect occurs [30]. It is possible that the higher effective concentration of primers and template molecules resulting from the low microwell volume increased the overall reaction efficiency and postponed the onset of the plateau phase until a higher molar yield was achieved. However, it is also possible that this effect was caused by the high concentration of *Taq* used in the PTPCR reactions, as elevated polymerase concentration has also been shown effective in delaying the plateau effect [30, 31]. Despite the high final concentration of amplicon obtained, the amplification efficiency over 40 cycles was low (44.3, 68.9 and 67.5% for the 26, 50 and 76 μm deep wells, respectively), but greatest in the 50 μm deep wells. It should be recognized, however, that cycle number optimization was not conducted; similar amplification yields could likely have been achieved with far fewer cycles, increasing the efficiency of the PTPCR amplification.

3.2 Solid-phase PTPCR in 76 pL microwells

The experimental strategy for clonal solid-phase PTPCR, starting with a single effective copy of a single-stranded DNA fragment, and finishing with a specific bead-immobilized DNA amplicon detected by fluorescent probe hybridization, is depicted in Fig. 4 and described in detail below. Stage 1: Each PicoTiterPlate™ well contains PCR reaction mix consisting of a single-stranded template molecule (either single-stranded and annealed to the DNA capture beads, as shown here, or free-floating in solution), Forward "F" (red) and Reverse "R" (blue) primers in solution, as well as R primers attached to a DNA capture bead. Solution phase primers are present in an 8:1 molar ratio, with the F primer in excess. Arrows indicate the 5' → 3' DNA orientation. Stage 2: The initial thermal cycle denatures the DNA template, allowing R primers in solution to bind to the complementary region on the template molecule. Thermostable polymerases initiate elongation at the primer site (dashed line), and in subsequent cycles, solution-phase exponential amplification ensues. Bead immobilized primers are not assumed to be major contributors to the amplification at this stage. Stage 3: Early-phase PCR. During early exponential amplification (1–10 cycles) both F and R primers amplify the template equally, despite an excess of F primers in solution. Stage 4: Mid-phase PCR. Between cycles 10 and 30, the R primers are depleted, halting exponential amplification. The reaction then enters an asymmetric amplification phase, and the amplicon population becomes increasingly dominated by F strands. Stage 5: Late-phase PCR. After 30–40 cycles, asymmetric amplification continues to increase the concentration of F strands in solution. Excess F strands, without R strand complements, begin to anneal to bead-immobilized R primers. Thermostable polymerases utilize the F strand as a template to synthesize an immobilized R strand of the amplicon. Stage 6: Final-phase PCR. Continued thermal cycling forces additional annealing to bead-bound primers. Solution-phase amplification may be minimal at this stage, but concentration of immobilized R strands continues to increase. Stage 7: The nonimmobilized F strand, complementary to the immobilized R strand, is removed by alkali denaturation. The DNA capture beads are now populated by single-stranded R strands of the amplicon. Stage 8: Fluorescently labeled probes (green bars) complementary to the R strand are annealed to the immobilized strand. Probes specific for particular strand sequences are labeled with unique fluorophores (not shown), resulting in a range of homogenous and heterogeneous fluorescent signals depending on the number of discrete templates amplified within a given PicoTiterPlate™ well.

Initially, fluorescently labeled probe specificity was confirmed by binding biotinylated Fragment A or Fragment B test DNA fragments to streptavidin Sepharose beads, loading the beads into a 50 μm deep PicoTiterPlate™ by centrifugation and hybridizing a mixed population of fluorescently labeled probes for the Fragment A and Fragment B fragments. No mixed signals or nonspecific hybridizations were observed; the beads with the Fragment A product displayed the 488 nm signal, while the Fragment B beads exhibited the 635 nm signal (Figs. 5A, B). Close examination of Figs. 5A and B reveals a few Fragment A beads in the Fragment B pad and *vice versa*. Given the purity of the signal displayed by these nomadic beads, it is likely that they are either the product of some cross contamination during the loading process, or were washed from one pad to the other during subsequent wash steps.

As indicated in Fig. 5C, the fluorescent probes detected successful solid-phase PTPCR amplification of both Fragment A and Fragment B templates. Unfortunately, detailed quantitative analysis of the fluorescence data was impossible. Unlike microarray data, where fluorescence intensities can be normalized under the assumption of equimolar probe concentrations, the signals generated by the hybridized probe depended on the relative efficiency of dye incorporation within the probes, the sensitivity of the reactions to unequal amounts of template DNA, as well as the total and relative amounts of amplified product present on each bead. In addition, it is likely that the amount of template generated and retained on the DNA capture beads varied from well to well, and the number of capture

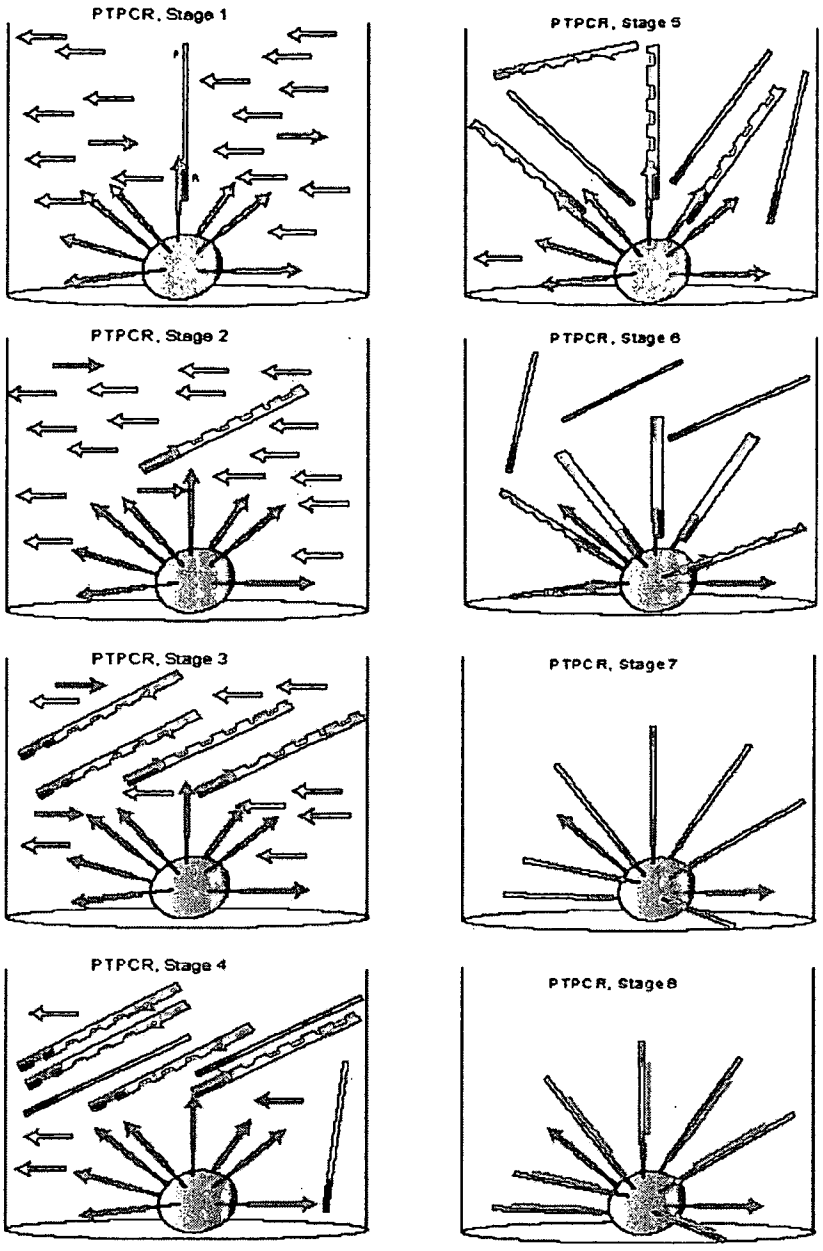

Figure 4. Schematic diagram of solid-phase PicoTiter Plate™ PCR. The cylindrical structures symbolize individual PicoTiter-Plate™ wells. Gray spheres symbolize beads with immobilized primers. Forward "F" (red) and Reverse "R" (blue) primers are shown in 5' → 3' orientation as indicated by arrows. Synthetized sequences complementary to the forward and reverse primers are shown as dark red (F complement) and dark blue (R complement) bars. Single-stranded template DNA is shown as solid gray line, newly synthetized DNA strands as dashed gray lines. Fluorescently labeled hybridization probes are shown as green bars.

primers bound to each bead is also likely to vary due to bead size distribution. As a result, the un-normalized ratios generated by the probe hybridization should be seen as semiqualitative rather than quantitative data. Nevertheless, the fluorescent signals generated by the hybridized probes ranged from a homogeneous Fragment B signal (red) to an equally homogenous Fragment A signal (green), with heterogeneous mixes of the two signals (degrees of yellow) evident as well. Due to the probe specificity displayed by the controls, as well as the sizeable number of homogenous red and green beads on the PicoTiterPlate™, it is unlikely that nonspecific probe hybridization caused the heterogeneous signals. The close proximity of homogenous beads of either template suggests it is unlikely that the heterogeneous beads resulted from amplicon leakage between wells during amplifica-

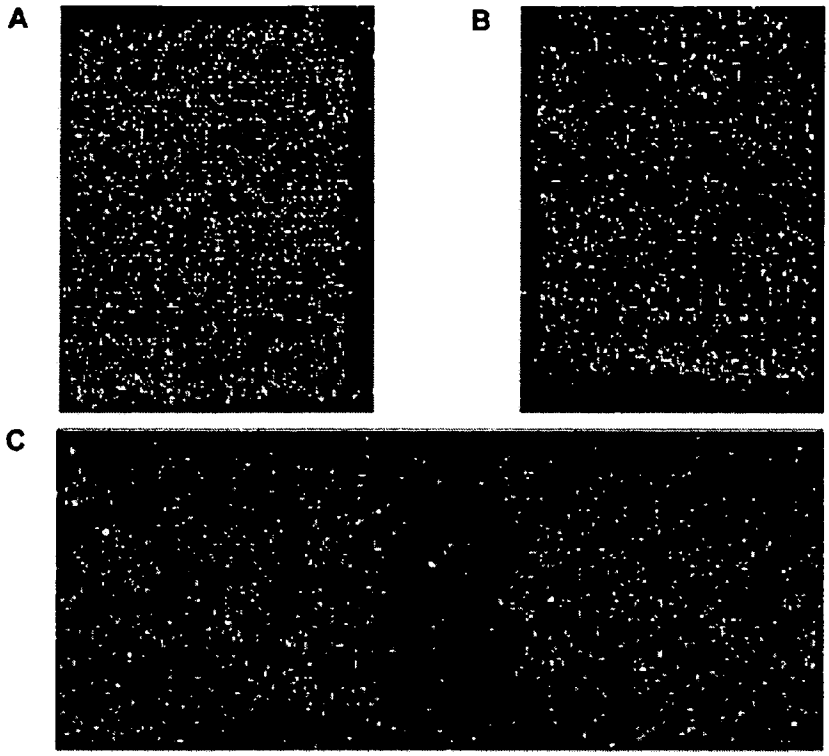

Figure 5. Fluorescent probe hybridization to bead-immobilized test DNA fragments. (A), (B) Specificity of a mixed population of probes hybridized to fragment A and fragment B immobilized on control beads, respectively. Fragment B beads demonstrate the Alexa Fluor 647 signal (shown as red), and the fragment A beads exhibit the Alexa Fluor 488 signal (green). (C) Probe fluorescence from DNA capture beads after PTPCR; beads display homogeneous fragment A and fragment B signals, as well as mixes of templates, shown as varying degrees of yellow.

tion; if intra-well cross-talk were responsible, one would expect to see heterogeneous beads located between homogenous beads of either template, and a generally patchy distribution of homogenous signals. Rather, it is likely that template molecules disassociated from their original bead and reannealed to new beads in the PicoTiterPlate™ loading mix prior to being spun into the microwells, or were washed from one bead to another as the PCR mix was applied to the PicoTiterPlate™. Regardless of the cause of the mixed template beads, the hybridization results show that PCR amplification in the PicoTiterPlate™ microwells can drive sufficient product to the DNA capture beads to enable fluorescent probe hybridization and detection.

4 Discussion

PCR has evolved into its current incarnation through a series of punctuated leaps in throughput. The original PCR was comprised of single tube reactions where the tubes were cycled by hand through different temperature water baths [1]. The advent of automated thermocyclers represented a major advance in the number of reactions that one individual could conduct; the subsequent development of 24-, 48-, 96-, and recently 384-well plates delivered dramatic increases in reaction throughput. The completion of the Human Genome Project, ahead of schedule and under budget [32], illustrates the importance of high capacity, low cost technology for genome-scale sequencing, above and beyond the current state of the technology. Further optimization of the DNA amplification process would be desirable due to cost of reagents, the sheer number of reactions, and the lengthy reaction times required. Our results demonstrate that PicoTiterPlate™-based PCR alleviates many of these factors, delivering another "evolutionary jump" in PCR technology. The microwells on a single PicoTiterPlate™ can function as up to 370 000 discrete reaction vessels achieving high yield (2.3×10^6 to 1.2×10^9-fold) amplification even at reaction volumes as low as 39.5 pL. As a result, throughput is increased, and the total reagent cost for PTPCR is reduced; the reaction volume contained in an entire 26 or 76 μm deep PicoTiterPlate™ is 15.3 and 43 μL, respectively. Increases in the size of the PicoTiterPlate™ can further increase the maximal throughput; for example, increasing the PicoTiterPlate™ dimensions to 40 mm × 75 mm provides approximately 1.4×10^6 discrete reaction vessels, and a PicoTiterPlate™ possessing

the same perimeter dimensions as a commercially available 96-well PCR plate (85.47 mm x 127.81 mm) could contain as many as 5.24×10^6 wells.

Low-volume PCR amplifications are not novel. Nagai *et al.* [14, 15] reported as many as 10000 simultaneous 85 pl PCR amplifications of a single PCR product. However, solution-phase PCR amplifications, regardless of the number and volume in which they are conducted, are of limited utility unless the product can be recovered easily and efficiently. Nagai *et al.* [15] required evaporation of the liquid reaction mixture, leaving the amplicon dried to the walls of the microreactor, after which it could be recovered for further manipulations. Our methodology avoids the problems of product recovery by including solid-phase amplification, immobilizing the PCR product to a DNA capture bead. Thus, the product of a PicoTiterPlate™ microwell reaction is not 370000 wells containing solution-phase PCR product, but up to 370000 beads bound with immobilized PCR product. These PCR products are suitable for numerous solid-phase methods of nucleic acid interrogation including the potential capacity to support a massively parallel approach to sequencing whole genomes containing up to hundreds of millions of bases. The simplicity of such a parallel processing method would drastically reduce costs for sequencing and other applications now requiring robotics to maintain large-scale cloning and PCR.

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Figure 1.

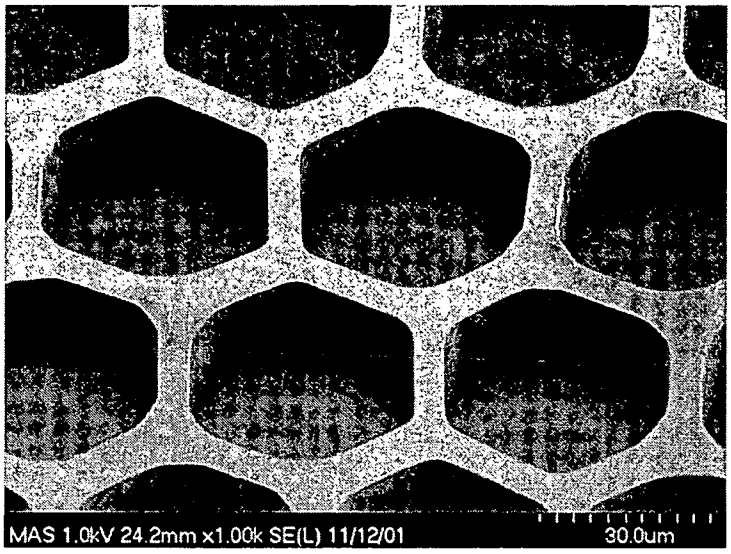


Figure 2.

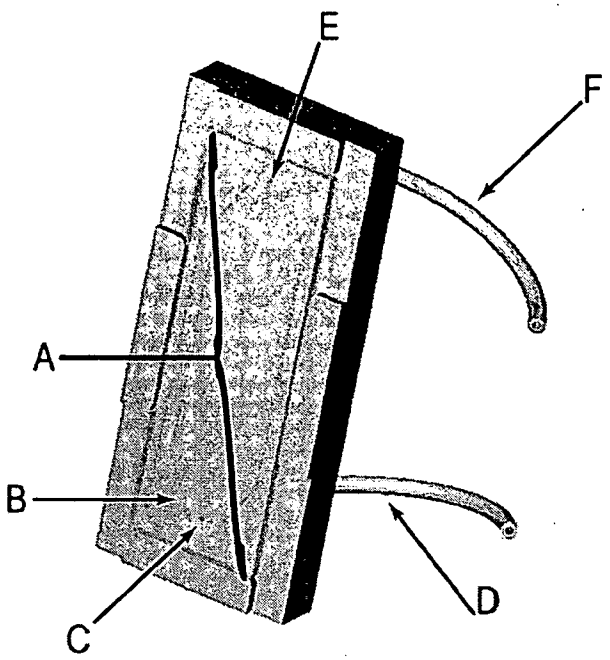
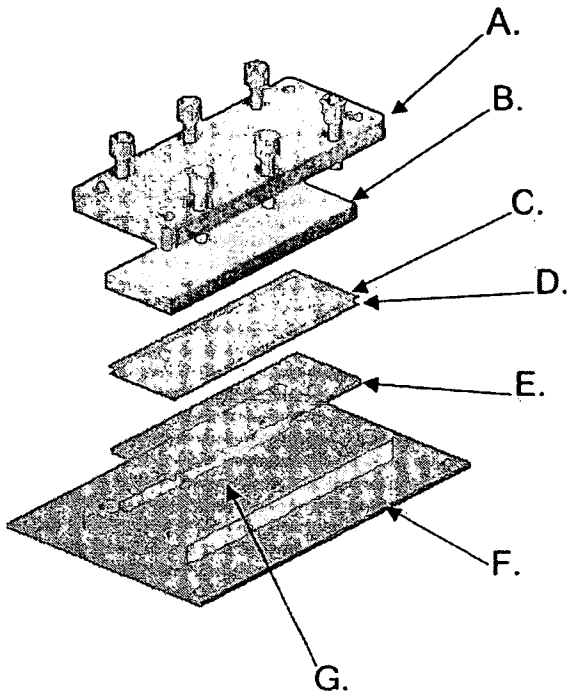
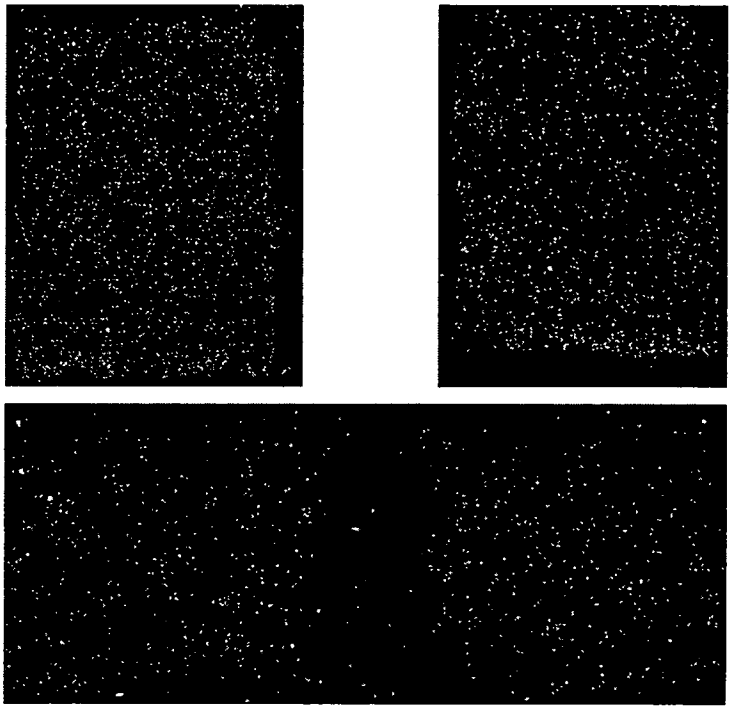


Figure 3.



56

Figure 4A - C.



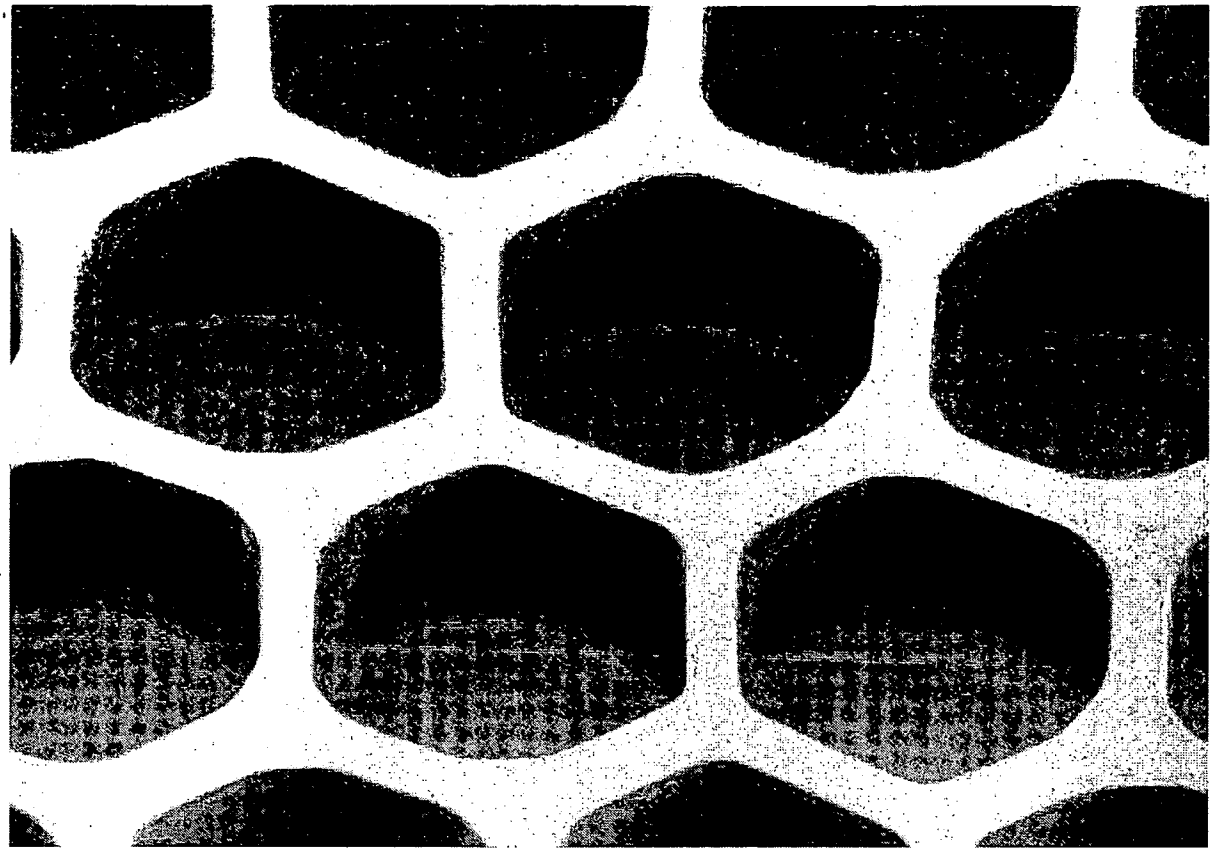
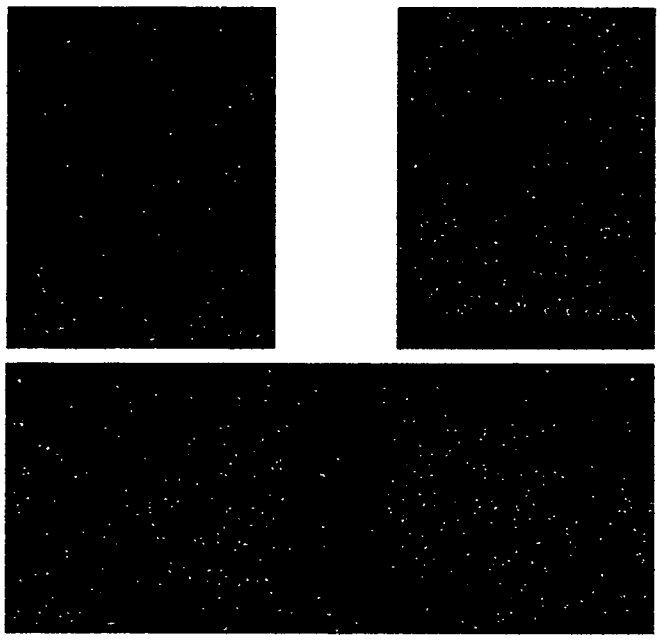
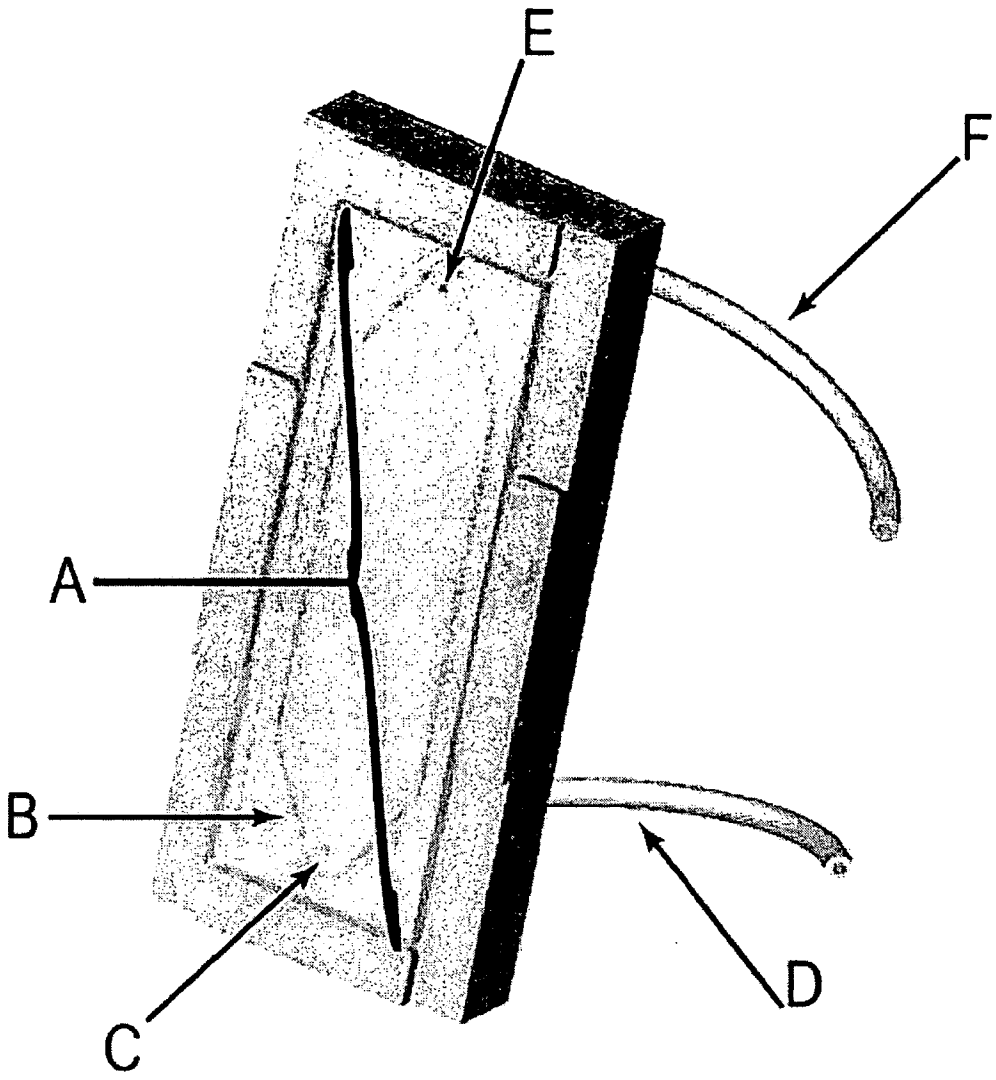


Figure 4A - C.




E
F
A
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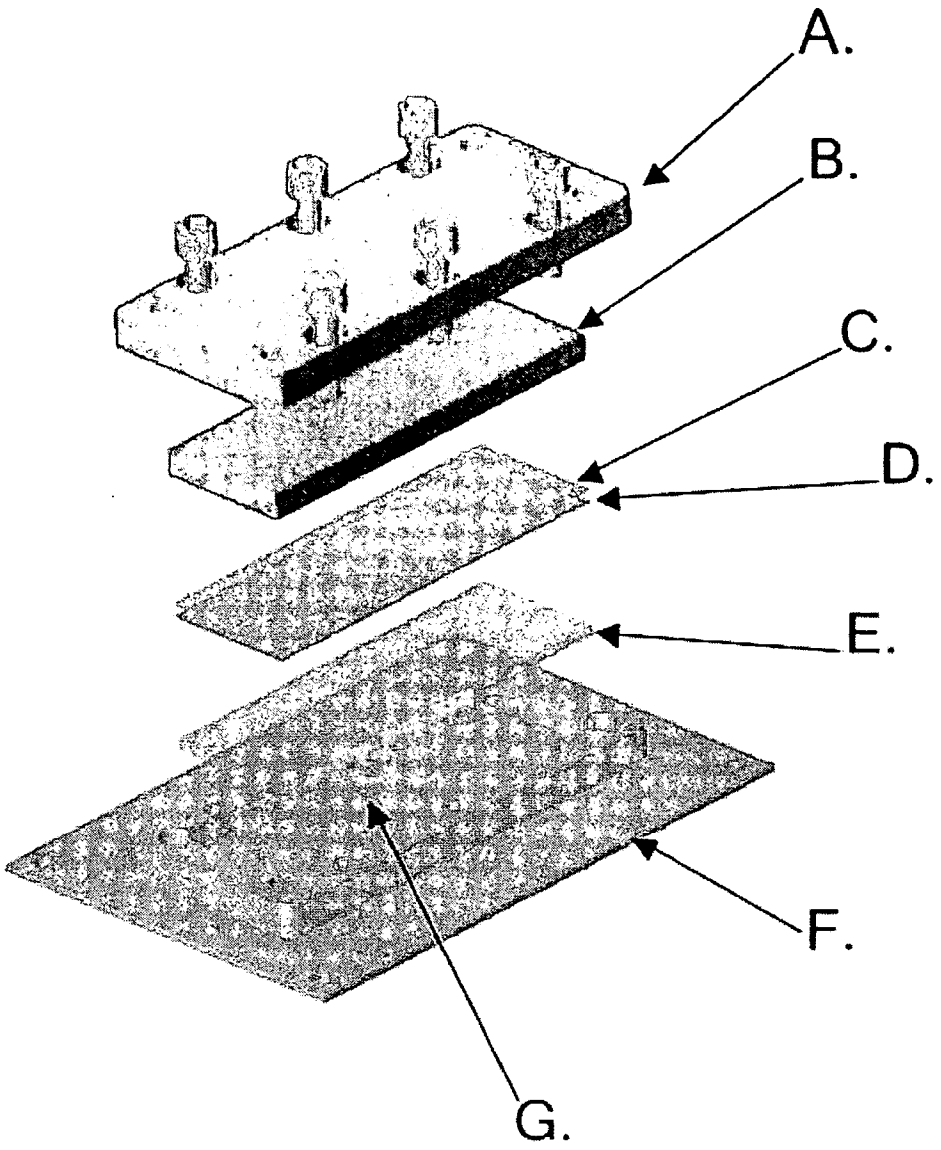
A.
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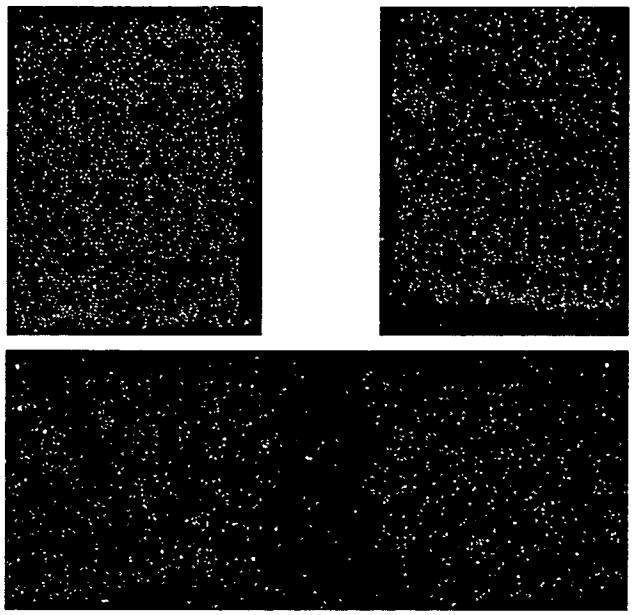
Figure 4A - C.

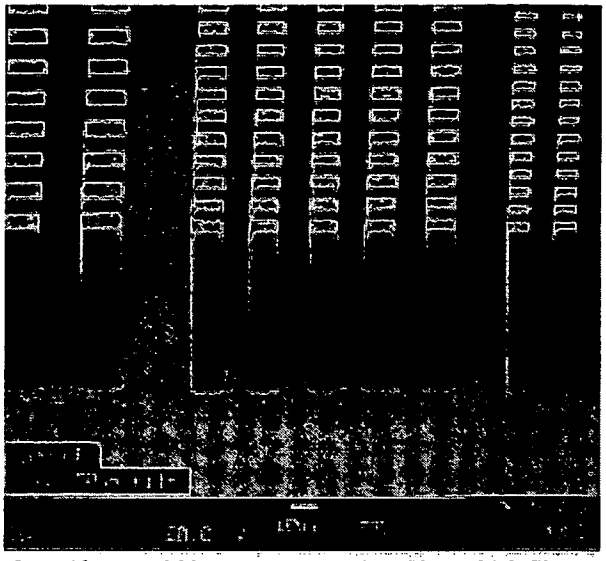


MICRO CHEM

NANO™ SU-8 2000

Negative Tone Photoresist Formulations 2002-2025

- High aspect ratio imaging
  Near vertical side walls
- Near UV (350-400nm) processing
- Improved coating properties
  Uniformity (lower surface tension)
  Adhesion
- Faster drying
  Improved throughput

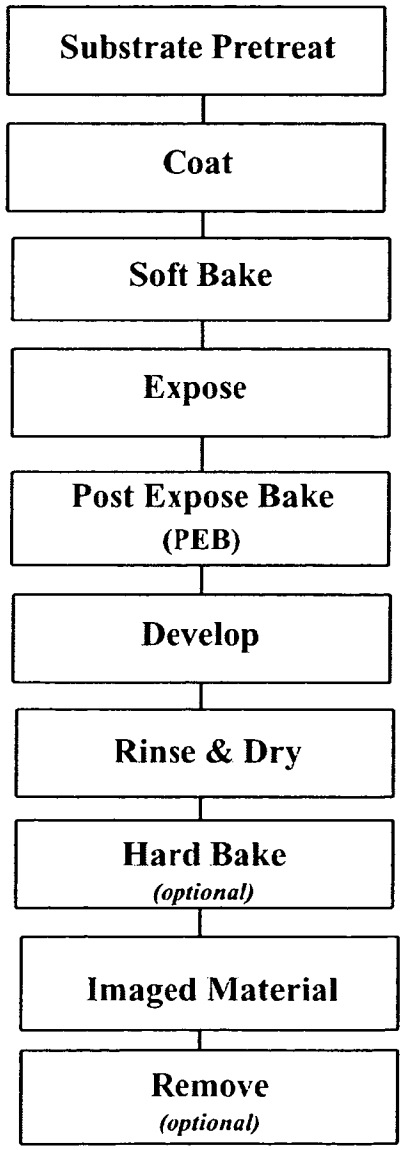


5µm, 10µm and 20µm post arrays in a 50µm thick film.

SU-8 2000 is a high contrast, epoxy based photoresist designed for micromachining and other microelectronic applications, where a thick, chemically and thermally stable image is desired. SU-8 2000 is an improved formulation of SU-8, which has been widely used by MEMS producers for many years. By using a faster drying, more polar solvent system, improved coating properties and higher throughput are realized. Film thicknesses of 0.5 to >200mm can be achieved with a single coat process. The excellent imaging characteristics of SU-8 are maintained. The exposed and subsequently cross-linked portions of the film are rendered insoluble to liquid developers. SU-8 2000 has very high optical transparency above 360nm, which makes it ideally suited for imaging near vertical sidewalls in very thick films. SU-8 2000 is best suited for permanent applications where it is imaged, cured and left in place

Process Guidelines

SU-8 2000 is most commonly processed with conventional near UV (350-400nm) radiation, although it may be imaged with e-beam or x-ray. i-line (365nm) is recommended. Upon exposure, cross-linking proceeds in two steps (1) formation of a strong acid during the exposure process, followed by (2) acid-initiated, thermally driven epoxy cross-linking during the post exposure bake (PEB) step.



A normal process is: spin coat, soft bake, expose, post expose bake (PEB) followed by develop. A controlled hard bake is recommended to further cross-link the imaged SU-8 2000 structures when they will remain as part of the device. The entire process should be optimized for the specific application. A baseline process is given here to be used as a starting point.

Substrate Pretreat

To obtain maximum process reliability, substrates should be clean and dry prior to applying the SU-8 2000 resist. Start with a solvent cleaning, or a rinse with dilute acid, followed by a DI water rinse. Where applicable, substrates should be subjected to a piranha etch / clean (H_2SO_4 & H_2O_2). To dehydrate the surface, bake at 200°C for 5 minutes on a contact hot plate or 30 minutes in a convection oven. Adhesion promoters are typically not required. For applications that require electroplating and subsequent removal of SU-8 2000 apply MicroChem's OmniCoat prior to processing.

Coat

SU-8 2000 resists are designed to produce low defect coatings over a very broad range of film thickness. The film thickness versus spin speed data displayed in Table 1 and Figure 1 provide the information required to select the appropriate SU-8 2000 resist and spin conditions, to achieve the desired film thickness.

The recommended coating conditions are:

(1) STATIC Dispense: Approximately 1ml of SU-8 2000 per inch of substrate diameter.
(2) Spread Cycle: Ramp to 500 rpm at 100 rpm/second acceleration. This will take 5 seconds.
(3) Spin Cycle: Ramp to final spin speed at an acceleration of 300 rpm/second and hold for a total of 30 seconds.

Product Name	Viscosity** (cSt)	Thickness** (µms)	Spin Speed (rpm)
		2	3000
SU-8 2002	7.5	2.5	2000
		3	1000
		5	3000
SU-8 2005	45	6	2000
		7.5	1000
		7	3000
SU-8 2007	140	8.5	2000
		12.5	1000
		10	3000
SU-8 2010	380	13	2000
		20	1000
		15	3000
SU-8 2015	1250	21	2000
		38	1000
		25	3000
SU-8 2025	4500	41	2000
		75	1000

Table 1. Thickness vs. spin speed data for selected SU-8 2000 resists.
*** Approximate*

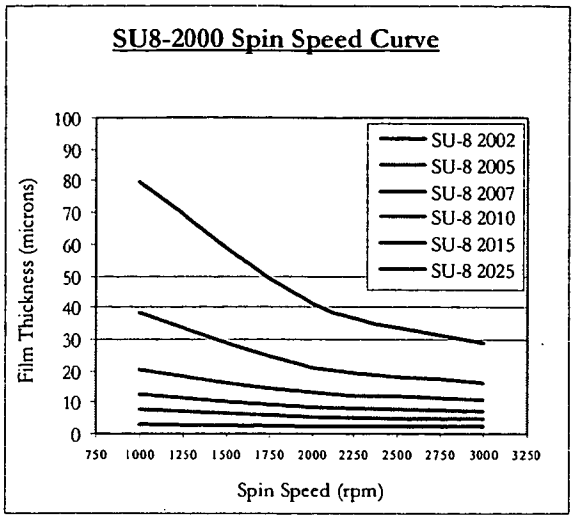


Figure 1. Spin speed vs. thickness curves for selected SU-8 2000 resists.

Soft Bake

After the resist has been applied to the substrate, it must be soft baked to evaporate the solvent and densify the film. SU-8 2000 is normally baked on a level hot plate, although convection ovens may be used. The following bake times are based on contact hot plate processes. Bake times should be optimized for proximity and convection oven bake processes since solvent evaporation rate is influenced by the rate of heat transfer and ventilation.

For best results, ramping or stepping the soft bake temperature is recommended. Lower initial bake temperatures allow the solvent to evaporate out of the film at a more controlled rate, which results in better coating fidelity, reduced edge bead and better resist -to-substrate adhesion. Refer to Table 2. for TWO STEP contact hot plate process recommendations.

Product Name	Thickness (µms)	Pre-bake @ 65°C	Softbake @ 95°C
	2	1	2
SU-8 2002	2.5	1	2
	3	1	2
	5	1	2
SU-8 2005	6	1	2
	7.5	1	2
	7	1	2
SU-8 2007	8.5	1	2
	12.5	1	2
	10	1	2
SU-8 2010	13	1	2
	20	1	3
	15	1	2
SU-8 2015	21	1	3
	38	2	5
	25	1	3
SU-8 2025	41	2	5
	75	3	9

Table 2. Recommended soft bake parameters

Expose

SU-8 is optimized for near UV (350-400nm) exposure. i-line exposure tools are recommended. SU-8 is virtually transparent and insensitive above 400nm but has high actinic absorption below 350nm. This can be seen in Figure 2. Excessive dose below 350nm may, therefore, result in over exposure of the top portion of the resist film, resulting in exaggerated negative sidewall profiles or T-topping. The optimal exposure dose will depend on film thickness (thicker films require higher dosage) and process parameters. The exposure dose recommendations in Table 3. are based on source intensity measurements taken with an i-line (365nm) radiometer and probe.

Expose tip: When using a broad spectral output source, for best imaging results, i.e. straightest sidewalls, filter out excessive energy below 350nm.

Catastrophic adhesion failure, severely negative sidewalls and excessive cracking often indicate an under cross-linking condition. To correct the problem, increase the exposure dose and/or increase the post exposure bake (PEB) time.

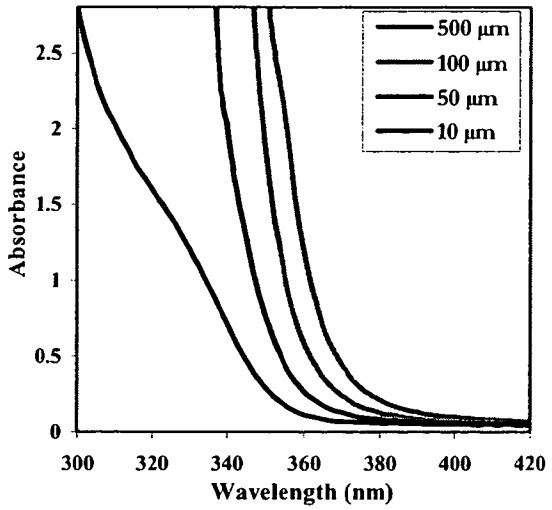


Figure 2. SU-8 absorbance vs. film thickness

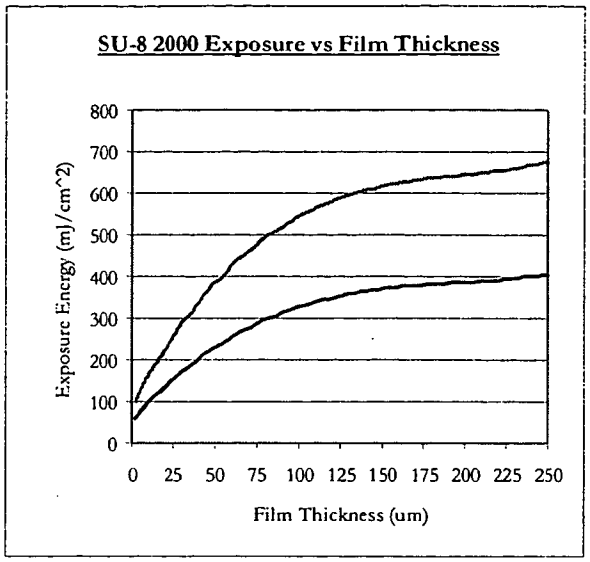


Table 3. Recommended expose dose processes

Post Expose Bake

Following exposure, a post expose bake (PEB) must be performed to selectively cross-link the exposed portions of the film. This bake can be performed either on a hot plate or in a convection oven. Optimum cross-link density is obtained through careful adjustments of the exposure and PEB process conditions. The bake recommendations below are based on results obtained with a contact hot plate.

PEB tip: SU-8 is readily cross-linked and can result in a highly stressed film. To minimize stress, wafer bowing and resist cracking, a slow ramp or TWO STEP contact hot plate process, as shown in Table 4., is recommended. Rapid cooling after PEB should be avoided.

Product Name	Thickness (µms)	PEB 1 @65°C	PEB 2 @95°C
	2	1	1
SU-8 2002	2.5	1	1
	3	1	1
	5	1	1
SU-8 2005	6	1	1
	7.5	1	1
	7	1	1
SU-8 2007	8.5	1	2
	12.5	1	2
	10	1	2
SU-8 2010	13	1	2
	20	1	2
	15	1	2
SU-8 2015	21	1	2
	38	1	3
	25	1	3
SU-8 2025	41	1	3
	75	1	7

Table 4. Recommended post expose bake parameters

Develop

SU-8 2000 resists have been optimized for use with MicroChem's SU-8 Developer. Immersion, spray or spray-puddle processes can be used. Other solvent based developers such as ethyl lactate and diacetone alcohol may also be used. Strong agitation is recommended for high aspect ratio and/or thick film structures. Recommended develop times are given in Table 5. for immersion processes. These proposed develop times are approximate, since actual dissolution rates can vary widely as a function of agitation rate, temperature and resist processing parameters.

Product Name	Thickness (µms)	Development (minutes)
	2	1
SU-8 2002	2.5	1
	3	1
	5	1
SU-8 2005	6	1
	7.5	1
	7	1
SU-8 2007	8.5	2
	12.5	3
	10	2
SU-8 2010	13	3
	20	3
	15	3
SU-8 2015	21	3
	38	4
	25	4
SU-8 2025	41	5
	75	7

Table 5. Recommended develop processes

Rinse and Dry

Following development, the substrate should be rinsed briefly with isopropyl alcohol (IPA), then dried with a gentle stream of air or nitrogen.

Rinse tip: If a white film is produced during rinse, this is an indication that the substrate has been under developed. Simply immerse or spray the substrate with SU-8 developer to remove the film and complete the development process. Repeat the rinse step.

Hard Bake (cure)

SU-8 2000 has good mechanical properties, therefore hard bakes are normally not required. For applications where the imaged resist is to be left as part of the final device, the resist may be ramp/step hard baked between 150-200°C on a hot plate or in a convection oven to further cross link the material. Bake times vary based on type of bake process and film thickness.

Removal

SU-8 2000, after expose and PEB, is a highly cross-linked epoxy, which makes it extremely difficult to remove with conventional solvent based resist strippers. MicroChem's Remover PG will swell and lift off minimally cross-linked SU-8 2000. However, if OmniCoat has been applied immersion in Remover PG should effect a clean and thorough Lift-Off of the SU-8 2000 Material. It will not remove fully cured or hard baked SU-8 2000 without the use of OmniCoat. Alternate removal processes include immersion in oxidizing acid solutions such as piranha etch / clean, plasma ash, RIE, laser ablation and pyrolosis.

To remove minimally cross-linked SU-8 2000, or if using Omnicoat, with Remover PG, heat the bath to 50-80°C and immerse the substrates for 30-90 minutes. Actual strip time will depend on resist thickness and cross-link density
For more information on MicroChem Omnicoat and Remover PG please see the relevant product data sheets.

Storage

Store SU-8 2000 resists upright in tightly closed containers in a cool, dry environment away from direct sunlight at a temperature of 40-70°F(4-21°C). Store away from light, acids, heat and sources of ignition. Shelf life is twelve months from date of manufacture.

Disposal

SU-8 2000 resists may be included with other waste containing similar organic solvents to be discarded for destruction or reclaim in accordance with local state and federal regulations. It is the responsibility of the customer to ensure the disposal of SU-8 2000 resists and residues made in observance all federal, state, and local environmental regulations.

Environmental, Health and Safety

Consult product Material Safety Data Sheet before working with SU-8 2000 resists. Handle with care. Wear chemical goggles, chemical gloves and suitable protective clothing when handling SU-8 2000 resists. Do not get into eyes, or onto skin or clothing. Use with adequate ventilation to avoid breathing vapors or mist. In case of contact with skin, wash affected area with soap and water. In case of contact with eyes, rinse immediately with water and flush for 15 minutes lifting eyelids frequently. Get emergency medical assistance.

The information is based on our experience and is, we believe to be reliable, but may not be complete. We make no guarantee or warranty, expressed or implied, regarding the information, use, handling, storage, or possession of these products, or the application of any process described herein or the results desired, since the conditions of use and handling of these products are beyond our control.

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Rev. 2/02

SHIPLEY

MICROPOSIT® S1800® SERIES PHOTO RESISTS

MICROPOSIT S1800 SERIES PHOTO RESISTS are positive photoresist systems engineered to satisfy the microelectronics industry's requirements for advanced IC device fabrication. The system has been engineered using a toxicologically safer alternative casting solvent to the ethylene glycol derived ether acetates. The dyed photoresist versions are recommended to minimize notching and maintain linewidth control when processing on highly reflective substrates.

MICROPOSIT S1800 SERIES PHOTO RESISTS FEATURE:

Product Assurance

- Lot-to-lot consistency through state-of-the-art physical, chemical and functional testing
- Filtered to 0.2 µm absolute

Coating Properties

- [1]Cellosolve® Acetate and xylene free
- Striation-free coatings
- Excellent adhesion
- Excellent coating uniformity
- A variety of standard viscosities are available for single-layer processing

Exposure Properties

- Optimized for G-Line exposure
- Effective for broad-band exposure
- Reflective notch and linewidth control using dyed versions

Develop Properties

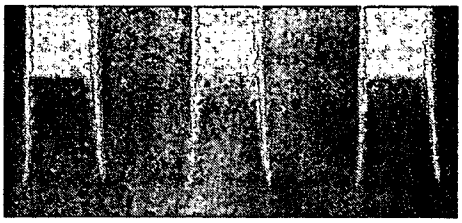
- Optimized for use with the MICROPOSIT® MF®-319 Metal-Ion-Free DEVELOPER family
- Compatible with Metal-Ion-Bearing MICROPOSIT DEVELOPERS

Removal Property

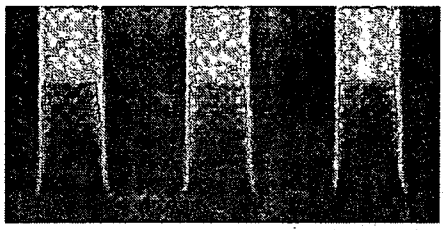
- Residue-free photoresist removal using standard MICROPOSIT REMOVERS

High Resolution Process Parameters (Refer to Figure 1)	
Substrate:	Polysilicon
Photoresist:	MICROPOSIT® S1813® PHOTO RESIST
Coat:	12,300Å
Softbake:	115°C/60 sec. Hotplate
Exposure:	Nikon 1505 G6E, G-Line (0.54 NA), 150 mJ/cm²
Develop:	MICROPOSIT® MF®-321 DEVELOPER 15 ÷ 50 sec. Double Spray Puddle (DSP) @ 21°C

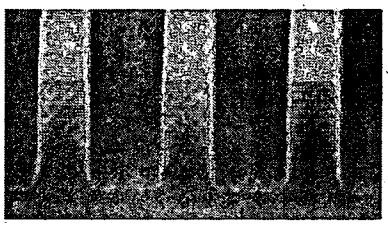
[1]Registered trademark of Union Carbide Corporation



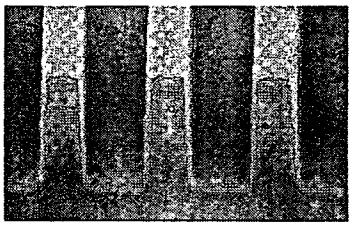
0.80 µm Lines/Spaces



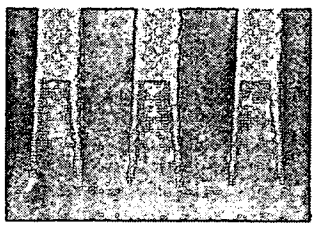
0.70 µm Lines/Spaces



0.60 µm Lines/Spaces



0.50 µm Lines/Spaces



0.48 µm Lines/Spaces

Masking Linearity SEMS
Figure 1.

MPR S1800
1093

Instructions for Use

The following instructions cover the use of MICROPOSIT S1800 SERIES PHOTO RESISTS for all levels of microelectronic device fabrication. Exact process parameters are application and equipment dependent.

Substrate Preparation

MICROPOSIT S1800 SERIES PHOTO RESISTS work well with the hexamethyldisilazane based MICROPOSIT PRIMERS. Concentrated MICROPOSIT PRIMER is recommended when vacuum vapor priming. Diluted PRIMER is recommended for liquid phase priming applications.

Coat

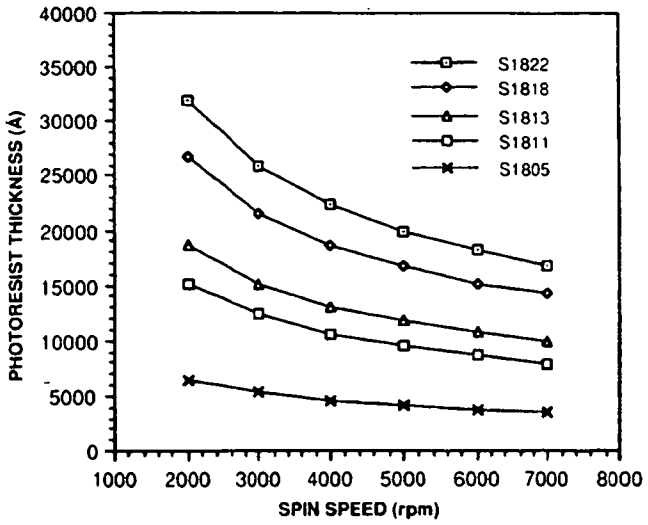
MICROPOSIT S1800 SERIES PHOTO RESISTS provide uniform defect-free coatings over a wide range of film thicknesses. The film thickness versus spin speed plots displayed in **Figures 1 and 2** provide the information required to properly select a MICROPOSIT S1800 PHOTO RESIST version to meet process dependent thickness specifications. Maximum coating uniformity is typically attained between the spin speeds of 3500 rpm and 5500 rpm.

Process Parameters (Refer to Figures 1 and 2)	
Substrate	Silicon
Coat	SVG 81
Softbake	115°C/60 seconds Hotplate
Measure	Nanometrics 210

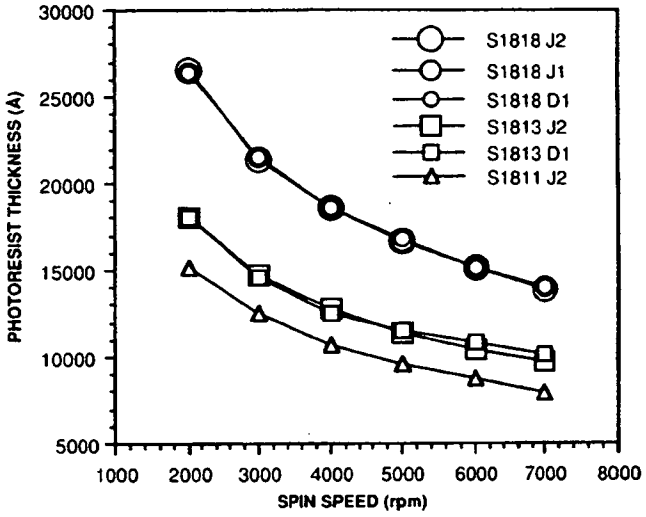
The dispersion curve and Cauchy equation displayed in **Figure 3** describe how the refractive index of the photoresist film varies as a function of the wavelength of light incident upon the film. This information is required to program ellipsometric and other optically based photoresist measuring equipment.

Process Parameters (Refer to Figure 3)	
Substrate	Silicon
Coat	13,675Å
Softbake	115°C/60 seconds Hotplate
Measure	Prometrix SM300

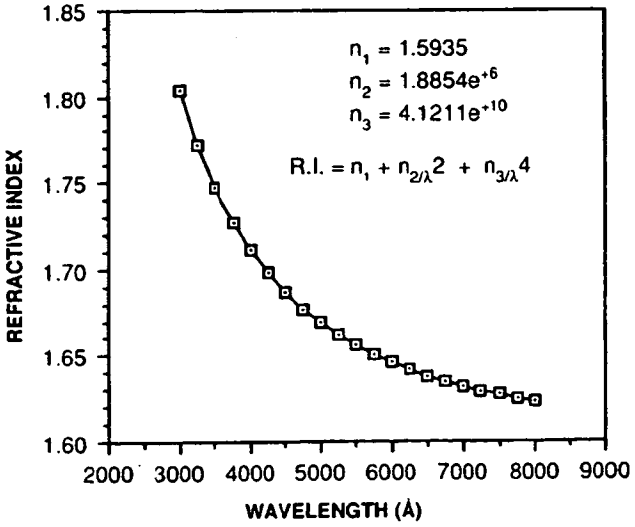
MICROPOSIT S1800 PHOTO RESIST UNDYED SERIES
Figure 1. Spin Speed Curves



MICROPOSIT S1800 PHOTO RESIST DYED SERIES
Figure 2. Spin Speed Curves



MICROPOSIT® S1813® PHOTO RESIST
Figure 3. Dispersion Curve



Exposure

Proper film thickness selection is critical in order to reduce photospeed and critical dimension variability. The interference curves displayed in **Figure 4** illustrate the photospeed variability as a function of film thickness. Dyed versions suppress the interference effects which are more pronounced when exposing with monochromatic light sources and when using reflective substrates.

Process Parameters (Refer to Figure 4)	
Substrate	Silicon
Coat	GCA 1006 [2] WAFERTRAC®
Softbake	115°C/60 seconds Hotplate
Expose	GCA 8500 G-Line (0.35 NA)
Developer	MF-321 / 10 + 30 DSP @ 21°C

MICROPOSIT S1800 SERIES PHOTO RESISTS can be exposed with light sources in the spectral output range of 350 nm - 450 nm. The exposure properties have been optimized for use at 436 nm. **Figures 5 and 6** show the absorbance spectrums for MICROPOSIT S1813 and S1813 J2® PHOTO RESISTS.

Process Parameters (Refer to Figures 5 and 6)	
Substrate	Quartz
Coat	12,300Å
Softbake	115°C/60 seconds Hotplate
Expose	Oriel Scanning Wedge
Measure	Hewlett Packard 8450A Spectrophotometer

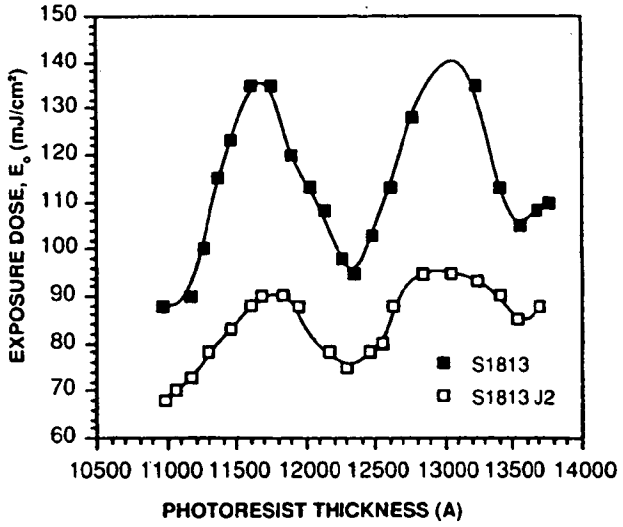
Table 1 summarizes the Dill parameters for each MICROPOSIT S1800 SERIES PHOTO RESIST version. Dill parameters are used in optical exposure models such as SAMPLE and PROLITH.

MICROPOSIT S1800 SERIES PHOTO RESISTS
Table 1. Dill Parameters

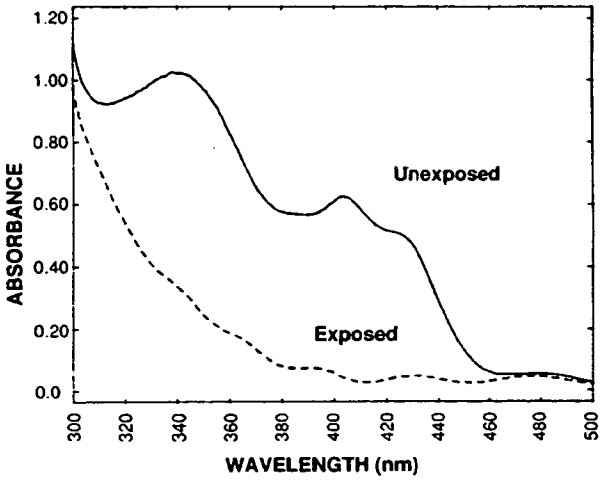
	365 nm		436 nm	
Photoresist	A (μm^{-1})	B (μm^{-1})	A (μm^{-1})	B (μm^{-1})
S1813	1.07	0.31	0.61	0.08
S1813 D1	1.05	0.34	0.58	0.26
S1811 J2	1.07	0.49	0.59	0.61
S1818 J1	1.06	0.42	0.57	0.37

Registered trademark of GCA, a unit of General Signal

MICROPOSIT S1813 and S1813 J2 PHOTO RESISTS
Figur 4. Interference Curv s



MICROPOSIT S1813 PHOTO RESIST
Figure 5. Absorbance Spectrum



MICROPOSIT S1813 J2 PHOTO RESIST
Figure 6. Absorbance Spectrum

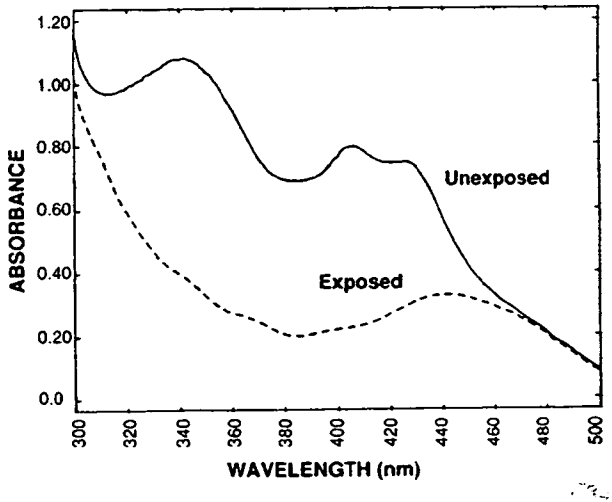


Figure 7 displays a contrast curve for MICROPOSIT S1813 PHOTO RESIST developed with MICROPOSIT® MF®-321 DEVELOPER. In general, high contrast values correlate to higher angle wall profiles.

Process Parameters (Refer to Figure 7)	
Substrate	Silicon
Coat	12,300Å
Softbake	115°C/60 seconds Hotplate
Expose	GCA 8500 G-Line (0.35 NA)
Develop	MF-321 / 10 + 30 DSP @ 21°C

DEVELOP

MICROPOSIT S1800 SERIES PHOTO RESISTS are compatible with both Metal-Ion-Free (MIF) and Metal-Ion-Bearing (MIB) developers. A photoresist and developer system is dependent upon specific application requirements. Contact your local Shipley Technical Sales Representative for additional product information.

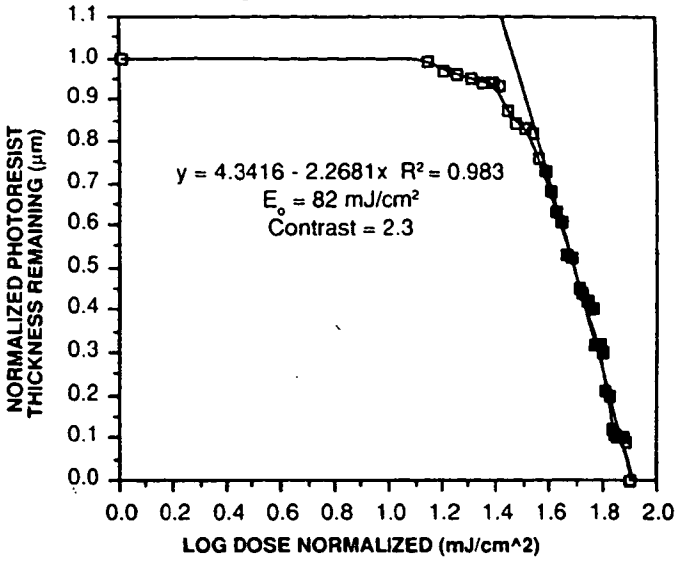
Figures 8 thru 10 illustrate the lithographic functionality of MICROPOSIT S1813 PHOTO RESIST using process parameters designed to maximize resolution while maintaining excellent exposure and focus latitude (refer to SEM photographs in **Figure 1**). The functional lithographic responses are summarized in **Table 2.**

Process Parameters (Refer to Figures 8 thru 10)	
Substrate	Silicon
Coat	12,300Å
Softbake	115°C/60 seconds Hotplate
Expose	Nikon 1505 G6E G-Line (0.54 NA)
Develop	MF-321 / 15 + 50 DSP @ 21°C

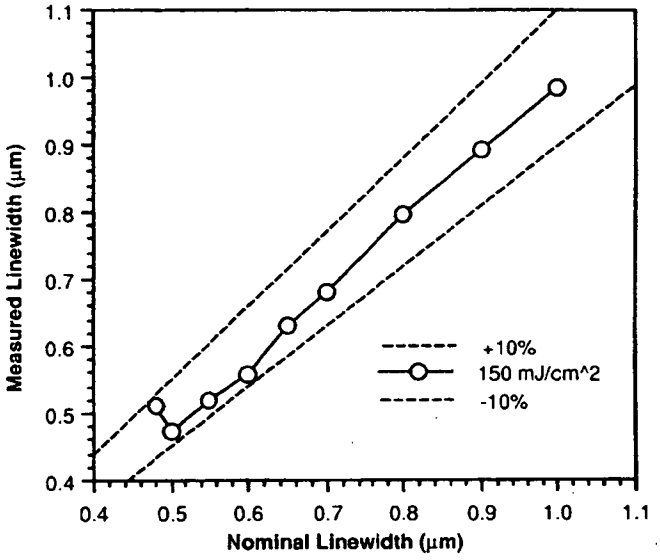
MICROPOSIT S1813 PHOTO RESIST
with MICROPOSIT MF-321 DEVELOPER
Table 2. Functional Lithographic Summary Data

Sizing Energy	150 mJ/cm² (1.3 E_o)	
Resolution	0.48 µm	
Masking Linearity (±10% CD)	0.50 µm	
	1.0 µm L/S	0.60 µm L/S
Exposure Latitude (±10% CD)	65%	45%
Focus Latitude (±10% CD) ≥ 85° Wall Angle	2.25 µm	1.25 µm

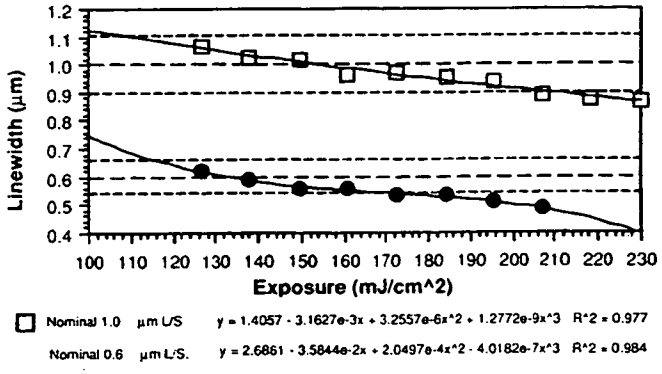
MICROPOSIT S1813 PHOTO RESIST
Figure 7. Contrast Curve



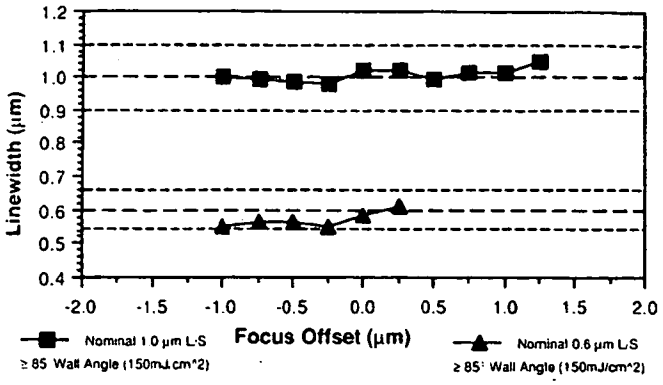
MICROPOSIT S1813 PHOTO RESIST
Figure 8. Masking Linearity Plot



MICROPOSIT S1813 PHOTO RESIST
Figure 9. Exposure Latitude Plot



MICROPOSIT S1813 PHOTO RESIST
Figure 10. Focus Latitud Pl t



Handling Precautions

WARNING: MICROPOSIT S1800 SERIES PHOTO RESISTS are combustible mixtures containing propylene glycol monomethyl ether acetate. Contact with eyes, skin and mucous membranes causes irritation. Handle with care. Do not get in eyes, on skin or on clothing. Avoid breathing vapors or mists. Use with adequate ventilation. Wash thoroughly after handling.

Wear chemical goggles, chemical gloves and suitable protective clothing when handling MICROPOSIT S1800 SERIES PHOTO RESISTS.

In case of eye or skin contact, flush affected areas with plenty of water for at least 15 minutes. Then contact a physician at once.

Consult product Material Safety Data Sheet before using.

Toxicological and Health Advantages

The solvent used in MICROPOSIT S1800 SERIES PHOTO RESISTS is propylene glycol monomethyl ether acetate. Toxicological studies reported that propylene glycol derivatives contained in MICROPOSIT S1800 SERIES PHOTO RESISTS do **not** demonstrate the adverse blood effects and reproductive effects that ethylene glycol derived ether acetates demonstrate (NIOSH Current Intelligence Bulletin 9 - 5/2/83).

Storage

Store MICROPOSIT S1800 PHOTO RESISTS only in upright, original containers in a dry area at 50°-70°F (10°-21°C). Store away from light, oxidants, heat, and sources of ignition. Do not store in sunlight. Keep container sealed when not in use.

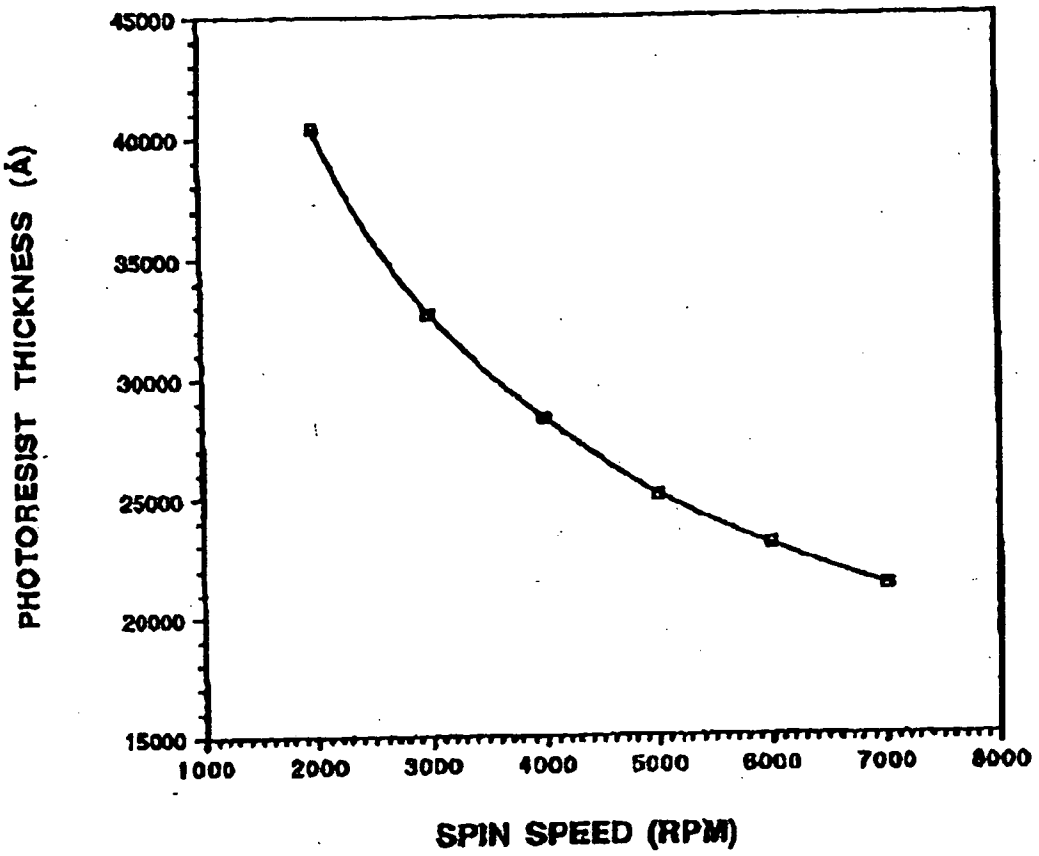
Equipment

MICROPOSIT S1800 SERIES PHOTO RESISTS are compatible with most commercially available photoresist processing equipment. Compatible materials include stainless steel, glass, ceramic, unfilled polypropylene, high density polyethylene, polytetrafluoroethylene, or equivalent materials.

Technical Literature

Please contact your Shipley Technical Sales Representative for information on the use and performance of Shipley products.

MEGAPOSIT SC1827 PHOTO RESIST
SPIN SPEED CURVE



PROCESS PARAMETERS:

SUB: 100 mm, 550cm
PRIME: 1.5 min. HMDS (vap.)
SPC: 100°C/60 secs.
TRACK: SVG 61
MET: NANOMETRICS 210
LOT: 8UL 003